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CYTOKINES, NUCLEOSOMES, AND LEUKOCYTE SIGNALING PROFILES IN PREDICTING DEVELOPMENT OF SEVERE ACUTE PANCREATITIS

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To my family

“Hard work always wins in the end”
– *Lucas Till*–

ABSTRACT

Acute pancreatitis (AP) is a common gastrointestinal disease of varying severity. While mild AP is a local inflammation of the pancreas that resolves within days, in severe AP (SAP) systemic inflammatory response is comparable to that seen in bacterial sepsis, leading to persistent organ dysfunction (OD), which is associated with substantial morbidity and mortality. However, in half of the SAP patients, the clinical signs of OD are not yet present on admission to hospital, potentially delaying the diagnosis of SAP and the initiation of maximal supportive care, thus worsening the prognosis.

The aims of this study were (i) to identify early predictive markers of SAP among patients with no OD on admission to hospital and (ii) to elucidate the aberrations in blood leukocyte signaling pathways in the early phase of AP and sepsis, which could reveal novel predictive markers of OD.

This clinical study consists of four prospective studies. All AP patients investigated were admitted to Helsinki University Hospital within 72 or 96 hours of onset of symptoms during the years 2003-2008 (Studies I and III), 2011-2014 (Study II), and 2010-2012 (Study IV). The fourth study includes also patients with sepsis. In the first study, the serum levels of 48 circulating cytokines were assessed on hospital admission in 163 AP patients using the Multiplex detection technique. Of SAP patients, 14/25 had no OD on admission. In the second study, the admission plasma levels of interleukin (IL)-8 and hepatocyte growth factor (HGF) were analyzed using cytokine-specific enzyme-linked immunosorbent assay (ELISA) in an independent cohort of 176 AP patients and 32 healthy controls. Of SAP patients, 10/23 had no OD on admission. In the third study, the admission plasma levels of nucleosomes were evaluated using ELISA in 74 AP patients. Of SAP patients, 14/24 had no OD on admission. In the fourth study, the phosphorylation of nuclear factor kappa B (NF- κ B), signal transducers and activators of transcription (STATs) 1 and 3, and extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinases (MAPK) were examined in appropriately stimulated or non-stimulated circulating leukocytes of 18 patients with AP, 14 patients with sepsis, and 28 healthy controls using phosphospecific whole-blood flow cytometry.

Our results show that IL-8, HGF, granulocyte colony-stimulating factor (G-CSF), and nucleosomes are associated with the severity of AP and predict development of SAP among AP patients without OD on admission. The result concerning IL-8 and HGF was confirmed in a second study, which also shows that among patients with OD on admission IL-8 may predict persistent OD, i.e. SAP. The discovered signaling aberrations in NF- κ B, STAT1, STAT3, and ERK1/2 MAPK pathways are largely similar in sepsis and SAP. However, only the results concerning STAT1 and STAT3 are associated with the severity of

AP. Additionally, STAT3 distinguishes patients with persistent OD (i.e. sepsis and SAP) from those without OD (i.e. mild and moderately severe AP).

In conclusion, circulating levels of IL-8 and HGF may serve as useful predictors of SAP in AP patients without OD on admission. Additionally, G-CSF and nucleosomes may predict development of SAP. Among patients with OD on admission, IL-8 may predict persistent OD. Signaling aberrations of circulating leukocytes in sepsis resemble those discovered in SAP. Aberrations in STAT1 and STAT3 pathways associate with the severity of AP and those in STAT3 with the presence of OD. Possibility that aberrations in STAT1 and STAT3 pathways provide novel markers for predicting development of OD warrants further studies. Early and accurate identification of patients at risk for SAP or OD may improve their prognosis. Additionally, such early markers may help to identify individual patients that will potentially benefit from immunomodulatory treatment modalities in the future.

TIIVISTELMÄ

Akuutti haimatulehdus on yksi yleisimmistä diagnooseista vatsaelinkirurgisella päivystysosastolla. Sen yleisimmät aiheuttajat ovat alkoholinkäyttö ja sappikivitauti. Vaikka suurin osa akuuteista haimatulehduksista rauhoittuu muutamassa päivässä, noin 20 % haimatulehduksista komplisoituu. Vaikeasta haimatulehduksesta puhutaan, kun sen aiheuttama elinvario kestää yli 48 tuntia. Kuolleisuus vaikeaan haimatulehdukseen on jopa 15-59 %.

Akuutti haimatulehdus aiheuttaa ensin haiman paikallisen tulehdusreaktion, joka vaikeassa haimatulehduksessa voimistuu ja etenee koko elimistön yleistyneeksi tulehdusreaktioksi aiheuttaen elinvarion esimerkiksi keuhkoihin tai munuaisiin. Systeeminen tulehdusreaktio ja sen aiheuttamat elinvariot kehittyvät vaikeassa haimatulehduksessa ainakin osin samoilla mekanismeilla kuin esimerkiksi sepsiksessä. Vaikeaa haimatulehdusta sairastavat potilaat hyötyvät voimien tarkasta monitoroinnista sekä mahdollisimman varhaisesta tehokkaan nestehoidon aloituksesta. Näiden potilaiden tunnistaminen pian oireiden alkamisen jälkeen on kuitenkin haastavaa, sillä vain noin puolella havaitaan kliiniset elinvarion merkit jo sairaalaan tullessa.

Tämän väitöskirjatyön tavoitteena oli löytää verestä merkkiaineita, joilla vaikean haimatulehduksen kehittyminen voitaisiin ennustaa jo taudin varhaisvaiheessa ennen elinvarion kliinisten merkkien ilmaantumista. Lisäksi selvitimme, tapahtuuko sepsispotilaiden veren valkosolujen signaalireiteissä vastaavia aktiivisuuden muutoksia kuin vaikeaa haimatulehdusta sairastavilla, ja voidaanko näiden avulla ennustaa elinvarion kehittymistä.

Tutkimus koostuu neljästä osatyöstä, joiden potilasrekrytointi suoritettiin Meilahden sairaalan päivystysalueella vuosina 2003-2008 (osatyöt I ja III), 2011-2014 (osatyö II) ja 2010-2012 (osatyö IV). Tutkimukseen otetuilla potilailla oli diagnosoitu akuutti haimatulehdus, ja oireiden alusta oli aikaa alle 72 tai 96 tuntia. Osatyössä I analysoitiin Multiplex-menetelmällä 48 sytokiinin seerumipitoisuudet 163 potilaalta, joista 25:lla oli vaikea haimatulehdus, mutta 14/25:lla ei ollut tulovaiheessa elinvariota. Osatyössä II analysoitiin ELISA-menetelmällä plasman interleukiini (IL) 8 -pitoisuus ja maksasolujen kasvutekijä (HGF) -pitoisuus uudessa potilasaineistossa ($n=176$; 10/23:lla vaikeista haimatulehduksista ei ollut tulovaiheessa elinvariota) sekä 32 terveellä verrokillä. Osatyössä III analysoitiin ELISA-menetelmällä plasman nukleosomipitoisuus 74 potilaalta (14/24:lla vaikeista haimatulehduksista ei ollut tulovaiheessa elinvariota). Osatyössä IV analysoitiin virtausytometriaan perustuvalla menetelmällä 18 haimatulehduspotilaan, 14 sepsispotilaan sekä 28 terveen verrokin verestä valkosolujen eräiden yleisimpien tulehdussignaalintireittien (NF- κ B, ERK1/2

MAP-kinaasi, STAT1 ja STAT3), aktiivisuus sekä aktiivisuuden muutokset solujen stimulaation jälkeen.

Tulokset osoittavat, että vaikeaa haimatulehdusta ennustavat ennen elinvarion merkkien ilmaantumista IL-8, HGF, granulosyyttikasvutekijä (G-CSF) sekä nukleosomit. Lisäksi potilailla, joilla on elinvario sairaalaan tullessa, IL-8 voi ennustaa elinvarion pitkittynyttä (≥ 48 tuntia) kestoaa. Havaitut muutokset tutkituissa valkosolujen signaalireittien aktiivisuudessa ovat samansuuntaisia sepsiksessä sekä vaikeaa haimatulehdusta sairastavilla. Kuitenkin vain muutokset STAT1 ja STAT3 signalointireiteissä näyttävät korreloivan akuutin haimatulehduksen vaikeusasteen kanssa. Lisäksi STAT3 voi olla käyttökelpoinen erottamaan elinvario-potilaat (sepsis tai vaikea akuutti haimatulehdus) ei-elinvario-potilaista (lievä tai keskivaikea akuutti haimatulehdus).

Yhteenvedona voidaan todeta, että IL-8 ja HGF toimivat kahdessa erillisessä potilasaineistossa vaikean haimatulehduksen ennustajina ennen elinvarion merkkien ilmaantumista, ja täten ne saattavat toimia käyttökelpoisina vaikean haimatulehduksen merkkiaineina taudin alkuvaiheessa. Näiden lisäksi G-CSF ja nukleosomit saattavat ennustaa vaikean haimatulehduksen kehittymistä ennen elinvarion merkkien ilmaantumista. Tutkituista valkosolujen signalointireiteistä STAT1 ja STAT3 korreloivat haimatulehduksen vaikeusasteen, ja STAT3 elinvarion, kanssa. Lisätutkimuksia tarvitaan selvittämään voisivatko STAT1 ja STAT3 toimia hyvin varhaisina elinvarion ennustajina. Varhaisten merkkiaineiden avulla voitaisiin löytää riskipotilaat ajoissa ja täten parantaa taudin ennustetta sekä löytää se potilasryhmä, joka voisi hyötyä tällä hetkellä vielä kokeellisesta immuunivasteeseen vaikuttavasta täsmähoidosta.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I Nieminen A, Maksimow M, Mentula P, Kyhälä L, Kylänpää L, Puolakkainen P, Kemppainen E, Repo H, Salmi M. Circulating cytokines in predicting development of severe acute pancreatitis. Crit Care 2014; 18 (3).

- II Penttilä AK, Lindström O, Hästbacka J, Kuuliala K, Mustonen H, Puolakkainen P, Kuuliala A, Salmi A, Hämäläinen M, Moilanen E, Repo H, Kylänpää L. Interleukin 8 and hepatocyte growth factor in predicting development of severe acute pancreatitis. Cogent Medicine 2017; 4: 1396634.

- III Penttilä AK*, Rouhiainen A*, Kylänpää L, Mustonen H, Puolakkainen P, Rauvala H, Repo H. Circulating nucleosomes as predictive markers of severe acute pancreatitis. J Intensive Care 2016; 4:14.
 *equal contribution

- IV Kuuliala K*, Penttilä AK*, Kaukonen KM, Mustonen H, Kuuliala A, Oiva J, Hämäläinen M, Moilanen E, Pettilä V, Puolakkainen P, Kylänpää L, Repo H. Signaling profiles of blood leukocytes in sepsis and in acute pancreatitis in relation to disease severity. Scand J Immunol. 2018; 87(2):88-98.
 *equal contribution

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ABBREVIATIONS

AP	Acute pancreatitis
AUC	Area under the curve
CARS	Compensatory anti-inflammatory response syndrome
CD	Cluster of differentiation
CECT	Contrast-enhanced computed tomography
CRP	C-reactive protein
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
ELISA	Enzyme-linked immunosorbent assay
ERCP	Endoscopic retrograde cholangiopancreatography
ERK	Extracellular signal-regulated kinase
G-CSF	Granulocyte colony-stimulating factor
HGF	Hepatocyte growth factor
HLA-DR	Human leukocyte antigen - antigen D related
HMGB1	High mobility group box 1 protein
ICU	Intensive care unit
IL	Interleukin
IL6R	Interleukin 6 receptor
I κ B	Inhibitor of kappa B
IQR	Interquartile range
JAK	Janus family of protein tyrosine kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MMS	Modified Marshall Score
MODS	Multiple organ dysfunction syndrome
MRI	Magnetic resonance imaging
NET	Neutrophil extracellular trap
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells

OD	Organ dysfunction
PAMP	Pathogen-associated molecular pattern
PMA	Phorbol-12-myristate-13-acetate
PRR	Pattern recognition receptor
RFU	Relative fluorescence unit
ROC	Receiver-operating characteristic
SAP	Severe acute pancreatitis
SIRS	Systemic inflammatory response syndrome
STAT	Signal transducer and activator of transcription
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor

1 INTRODUCTION

Acute pancreatitis (AP) is a common gastrointestinal inflammatory disease induced by toxic factors such as alcohol or biliary stones. The early events that take place in the pancreas include early activation of pancreatic proteases, resulting in acinar cell injury and subsequent release of damage-associated molecular patterns (DAMPs) that activate the innate immune system (Kang et al. 2014a). In parallel with these events, the nuclear factor kappa B (NF- κ B) pathway is activated in the pancreas, leading to production of proinflammatory cytokines and chemokines (Rakonczay et al. 2008). Consequently, recruitment of monocytes, neutrophils, and lymphocytes into the pancreas occurs.

The severity of AP varies from mild to severe. In severe AP (SAP), the initial local inflammatory reaction amplifies and spreads through the circulation to produce a severe systemic response complicated by organ dysfunction (OD) that is associated with substantial morbidity and mortality (Norman 1998, Kylänpää et al. 2012). It has been shown that the evolution of systemic inflammation is similar in SAP and sepsis, with a similar pattern of released inflammatory mediators and comparable clinical symptoms, which are a consequence of an uncontrolled acute inflammatory response of the host (Deitch 1992, Wilson et al. 1998). Simultaneously with the proinflammatory response, an anti-inflammatory response ensues, which may lead to excessive immune suppression, complicating the course of both diseases (Kylänpää et al. 2012, Hotchkiss et al. 2013).

In SAP, the evolution of systemic inflammation and subsequent development of multiple organ dysfunction syndrome (MODS) may occur rapidly, within the first few days or even hours (McKay and Buter 2003). Therefore, identifying AP patients who will develop SAP but who do not have clinical signs of OD on admission to hospital is crucial to minimize the delay in initiating optimal supportive treatment and intensive monitoring, which may improve their prognosis (Haydock et al. 2013). In the future, these SAP patients may also be an optimal target for immunomodulatory treatment modalities. However, predicting SAP on admission is complex. If a patient presents early after symptom onset, it is possible that OD signs have not yet developed. On the other hand, not all patients who present with OD will develop SAP (Wilson et al. 1990, Buter et al. 2002). Therefore, a reliable laboratory marker or a combination of markers is needed to support the clinical judgment.

The main purpose of the present investigation was to identify predictors of SAP in AP patients without OD on admission. Although predictive markers of SAP have been assessed extensively (Brivet et al. 1999, Mentula et al. 2005, Aoun et al. 2009), as a novel approach, we focused on AP patients showing no signs of OD on admission, which we think, reveals the true ability of the

markers to predict SAP. In more detail, we evaluated 48 circulating cytokines as well as circulating nucleosomes on admission to hospital as potential biomarkers in predicting development of SAP. Additionally, the ability of the markers to predict persistent OD, i.e. SAP, in patients with OD on admission was analyzed. Finally, in search for potential early predictors of OD, the aberrations in the activity of the major inflammation-associated leukocyte signaling pathways, including NF- κ B, signal transducers and activators of transcription (STATs) 1 and 3, and extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinases (MAPK), during AP and sepsis were investigated to determine whether the aberrations are similar in sepsis and SAP and whether they are associated with the severity of AP and the presence of OD.

2 REVIEW OF THE LITERATURE

2.1 EPIDEMIOLOGY AND ETIOLOGY OF ACUTE PANCREATITIS

Worldwide, the incidence of AP is increasing (Hamada et al. 2014, Krishna et al. 2017, Roberts et al. 2017), and in the United States AP is the third most common reason for hospital admission among gastrointestinal problems (Peery et al. 2015). The incidence of AP varies across countries and different regions. Among 17 European countries, the incidence ranges from 4.6 to 100 per 100 000 and is highest (> 40 per 100 000) in eastern and northern countries and lowest in Albania (Roberts et al. 2017). High incidence rates (> 40 per 100 000) have also been reported in USA (Frey et al. 2006), Japan (Hamada et al. 2014), and Taiwan (Shen and Lu 2011). The incidence in Finland, based on a study from 1989 in the Tampere region, is as high as 73 per 100 000 (Jaakkola and Nordback 1993).

Equal proportions of men and women develop AP, and the risk of AP progressively increases with **age**, but age and sex distributions differ based on etiology. Of lifestyle factors, **alcohol consumption** and **smoking** are associated with an elevated risk of AP, and **obesity** increases both the risk and severity of AP (Yadav and Lowenfels 2013). The proportions of different etiologic factors vary across countries and regions, but the three most common etiologic factors of AP are alcohol consumption, **gallstones**, and **idiopathic AP**. In European countries, gallstones are the underlying cause in 19-65% of cases and alcohol in 4-56% of cases (Roberts et al. 2017). In Finland, alcohol is the most common etiology in more than half of the episodes, whereas gallstones explain 20% (Mentula et al. 2003, Khan et al. 2013). However, the risk of biliary pancreatitis is not more than 2% in patients with asymptomatic gallstones, and the risk of alcoholic pancreatitis in heavy drinkers is unlikely to exceed 2-3% (Lankisch et al. 2002). Therefore, other factors, possible **genetic**, are also involved in triggering AP (Whitcomb 2013).

Other known etiologic factors are rare and include **medical treatments**, with more than 130 drugs reported to be associated with AP. However, the true causal role is lacking in the vast majority of drugs, and therefore, before suggesting a drug as the cause of AP, a careful evaluation of more common causes is recommended (Tenner 2014). **Hypertriglyceridemia** should be suspected in a patient with known genetic abnormality of lipoprotein metabolism or presenting with secondary factors such as uncontrolled diabetes, alcoholism, use of medications known to cause hypertriglyceridemia, and the third trimester of pregnancy. Triglyceride level ≥ 13 mmol/L indicates a high degree of suspicion of hypertriglyceridemia-induced AP (Scherer et al. 2014). The triglyceride level should be determined within 24 hours of presentation since fasting lowers the levels quickly. **Hypercalcemia** caused

by e.g. hyperparathyroidism, malignant diseases, and overdose of vitamin D or calcium is associated with AP, and therefore, calcium levels should be determined on admission (Kemppainen and Puolakkainen 2007). **Autoimmune pancreatitis** has typical morphologic features in contrast-enhanced computed tomography (CECT) and is usually an issue in the differential diagnosis between pancreatic tumor and chronic pancreatitis, rather than AP (Okazaki 2002). Especially in older patients with an unknown etiology of AP, the possibility of a **tumor** obstructing the ampullary region must be considered (Mujica et al. 2000).

Pancreas divisum, an anatomic variation of pancreatic duct resulting from the failure of fusion of the dorsal and ventral pancreatic buds during gestation, has been reported in 5-7% of the general population and is associated with an increased risk of AP. According to current knowledge, pancreas divisum does not cause AP alone, but is associated with genetic mutations, producing a cumulative effect (Bertin et al. 2012). Another much debated underlying factor is **Sphincter Oddi's dysfunction** (Cote et al. 2012, Romagnuolo 2013).

The risk of AP is also associated with **invasive procedures**. The frequency of AP after endoscopic retrograde cholangiopancreatography (ERCP) is 3.5% in unselected patients. Definite patient-related risk factors include suspected sphincter of Oddi dysfunction and female sex (Dumonceau et al. 2010). The risk is 0.85% after endoscopic ultrasound-guided fine needle aspiration of solid pancreatic mass (Eloubeidi et al. 2006) and up to 1% after single-balloon or double-balloon enteroscopy. It is of note that unspecific hyperamylasemia is seen in 16-17% of cases after single-balloon or double-balloon enteroscopy, probably due to repeated stretching of the small bowel or mesenteric ligaments (Lankisch et al. 2015). In addition, AP is associated with the **postoperative phase of surgery**. **Abdominal trauma**, mild, blunt, or sharp, may cause AP. Similarly, AP may follow **abdominal operations**, and an association between **cardiac surgery** and AP has been noted (Kemppainen and Puolakkainen 2007, Forsmark et al. 2016). Certain **viruses** (cytomegalovirus, mumps, Epstein-Barr virus) and **parasites** are also rare etiologic factors.

Finally, in many cases the etiology of AP remains unknown and is defined as idiopathic. The acceptable rate of idiopathic AP is < 20% (Working Party of the British Society of Gastroenterology 2005).

2.2 PATHOGENESIS AND PATHOPHYSIOLOGY OF ACUTE PANCREATITIS

2.2.1 TRIGGERING FACTORS

Mechanisms by which different etiologic factors, such as alcohol and gallstones, induce pancreatic cell injury and initiate AP include multiple pathobiologic pathways in acinar cells, but recent studies show also ductal cells as important participants in AP (Hegyi et al. 2011, Hegyi and Rakonczay 2015). However, the exact pathogenetic mechanisms are not fully understood.

Ethanol is metabolized via oxidative and non-oxidative pathways. Although the products of ethanol metabolism (acetaldehyde, oxidative stress, and fatty acid ethyl esters) have the capacity to injure the pancreas, recent studies show that especially fatty acid ethyl esters, the metabolite of the non-oxidative pathway, play a critical role in mediating alcohol-related pancreatic injury and inflammation (Apte et al. 2010, Hegyi et al. 2011). Within pancreatic ducts alcohol increases the formation of protein plugs that enlarge and form calculi leading eventually to acinar cell atrophy and fibrosis. Alcohol exerts toxic effects also on pancreatic stellate cells (Apte et al. 2010).

Transient pancreatic duct outflow obstruction is currently considered the initiating factor in biliary AP (Lerch et al. 1994). Duct obstruction may originate from refluxed bile acids, pancreatic duct hypertension, and/or aberrant acinar cell secretion (Lightner and Kirkwood 2001). Additionally, bile acids have direct toxic effects on acinar cells, where they elicit intracellular calcium release and subsequent cell injury and inflammation (Voronina et al. 2002, Hegyi et al. 2011). Bile acids can be taken up by acinar cells from the pancreatic duct through sodium-dependent co-transporters or G-protein-coupled bile acid receptor 1 (Kim et al. 2002, Perides et al. 2010), and from serum or interstitium via bicarbonate-dependent bile acid exchangers on the basolateral acinar cell surface (Kim et al. 2002).

2.2.2 INTRA-ACINAR EVENTS

Pathologic calcium signaling

Despite the initiating factor, an excessive rise in the cytoplasmic calcium concentration has been hypothesized to function as a trigger for the initiation of AP. While physiologic calcium spikes that regulate normal acinar cell functions are transient, pathologic sustained calcium release occurs during the early phase of AP. Sustained intracellular calcium release originates from the apical endoplasmic reticulum stores and acidic intracellular calcium stores, resulting in their sustained calcium depletion, which is followed by an excessive calcium entry from the interstitial fluid via the activation of the plasma membrane store operated calcium entry channel (Parekh and Putney

2005, Gukovskaya et al. 2016). Calcium overload leads to endocytic vacuole formation, adenosine triphosphate depletion, oxidative stress, and mitochondrial dysfunction, which further mediate acinar cell death pathways (Booth et al. 2011, Voronina et al. 2015, Mukherjee et al. 2016).

Premature trypsinogen activation

The traditional theory of the pathogenesis of AP is based on autodigestion caused by premature activation of trypsinogen to trypsin within the acinar cell (so-called trypsin-centered theory, first proposed by Chiari in 1896). In a normal physiologic condition, trypsinogen and other pancreatic proteases are produced and secreted as inactive zymogen granules that are only activated after they reach the duodenum. Digestive enzyme secretion is mediated by transient calcium spikes localized to the apical granular area. In AP apical exocytosis of zymogen granules is inhibited. Due to their altered intracellular trafficking, zymogens and lysosomal hydrolases, such as cathepsins, become co-localized into intra-acinar cell cytoplasmic vacuoles via a process called chrinophagy (Steer and Meldolesi 1987). Within these vacuoles, the lysosomal hydrolases activate trypsinogen, and trypsin further activates the other zymogens. The organelles containing activated zymogens become fragile and release their contents inside the acinar cell. Consequently, acinar cell injury/death pathways are activated (van Acker et al. 2006).

Local activation of NF- κ B

Recent data suggest that intra-acinar NF- κ B activation occurs very early in experimental AP independently of, yet concurrently with, trypsinogen activation (Gukovsky et al. 1998, Hietaranta et al. 2001). The NF- κ B pathway is one of the key inflammatory pathways mediating the expression of a large number of genes and subsequent pro- and anti-inflammatory cytokine production, and its activation increases the severity of experimental AP (Rakonczay et al. 2008, Huang et al. 2013).

2.2.3 INNATE AND ADAPTIVE IMMUNE RESPONSES

Inflammation, a tightly regulated complex network of different humoral and cellular responses, is a protective response to harmful stimuli such as microbial pathogens or damaged cells. While its ultimate aim is to eliminate the initial cause of cell injury, clear out damaged cells, and initiate tissue repair, an excessive and uncontrolled inflammatory response (such as in SAP or sepsis) is detrimental to the host, as is also insufficient inflammation. The immune system may be divided into innate and adaptive responses that work in close collaboration. The main components and functions of the innate and adaptive immune systems are presented in Table 1.

Table 1. *Comparison between innate and adaptive immune responses*

	Innate immune response	Adaptive immune response
Components	Physical and chemical barriers Granulocytes Monocytes/macrophages Dendritic cells Natural killer cells Mast cells Plasma proteins (complement)	Humoral immunity (B lymphocytes) Cell-mediated immunity (T lymphocytes, natural killer cells)
Age	Fully mature at birth	Immature at birth
Response	Immediate response	Delayed response over 1-2 weeks
Actions	Barrier functions Phagocytosis Cytotoxic effects Activation of inflammatory response Production of inflammatory mediators Activation of adaptive immune system (e.g. antigen presentation)	Specific antibody production Activation of phagocytic cells Cytokine production Cytotoxic effects Controlling immune tolerance Production of memory cells
Specificity	General, recognizes PAMPs and DAMPs via fixed set of receptors	Recognizes highly specific antigens through specific receptors
Memory	Short-lived	Long-term (development of memory cells)

Abbreviations: DAMP, Damage-associated molecular pattern; PAMP, Pathogen-associated molecular pattern.

While the innate immune system provides immediate but unspecific defense, the adaptive immune system is more sophisticated by offering targeted defense, but it reacts with a delay of some 1-2 weeks. The innate immune system recognizes conservative structures of foreign danger molecules through pattern recognition receptors (PRRs). Such danger molecules comprise pathogen-associated molecular patterns (PAMPs), which are derived from invading pathogens, and DAMPs, which are induced as a result of endogenous stress (Shi et al. 2003). Of the PRRs, the Toll-like receptors (TLRs) are the best-characterized family. Activation of TLRs activates downstream signaling pathways, such as NF- κ B, MAPK, and STATs, leading to increased transcription of inflammatory genes and subsequent production of proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL) 1 (Chen and Nunez 2010).

The adaptive immune system recognizes highly specific antigens, presented by specialized antigen-presenting cells, through specific receptors.

When activated, the cells of the adaptive immune system, namely T and B cells, provide targeted responses against the invading pathogen.

Traditionally, only adaptive immunity has been thought to be responsible for building immunological memory through production of memory cells during primary response. As a result, the immune system responds more rapidly and effectively to pathogens that have been encountered previously. However, also the cells of the innate immune system, such as macrophages, monocytes, and natural killer cells, show enhanced responsiveness when they re-encounter pathogens. This phenomenon is called “trained immunity” or “innate immune memory” that is shorter lived and a result of epigenetic reprogramming, i.e. it does not involve permanent genetic changes (Netea et al. 2016).

2.2.3.1 Inflammatory cells

Humoral inflammatory mediators activate and recruit circulating inflammatory cells to the site of inflammation. Leukocyte adhesion to the endothelium is essential for the development of an appropriate immune response. Neutrophils are the first recruited cells, following infiltrating monocytes/macrophages and lymphocytes.

Neutrophils

Recruitment of neutrophils to the inflammatory site is one of the hallmarks of the early phase of inflammation. Neutrophils are produced in the bone marrow, and in the steady state they circulate in the blood for a few hours, after which they undergo apoptosis. Apoptotic neutrophils are engulfed by macrophages and dendritic cells, which further regulates the neutrophil production in the bone marrow. Upon inflammatory reaction, granulocyte colony-stimulating factor (G-CSF) is essential for enhancing neutrophil production to meet the increased need, and neutrophils expand their life span in the circulation by several days (Lieschke et al. 1994, Borregaard 2010). To arrive at the site of inflammation, the neutrophils must cross the vascular wall.

The vascular endothelium is activated by proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-17, which results in enhanced expression of P- and L-selectin, integrins such as CD11b/CD18, and the immunoglobulin superfamily proteins intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 (Repo and Harlan 1999). As a result, stepwise adhesion (initial attachment, rolling, firm adhesion) of neutrophils to the vascular walls and their transendothelial migration occurs (Repo and Harlan 1999, Borregaard 2010). The activated endothelial wall secretes also e.g. IL-8, which activates additional neutrophils (Borregaard 2010). The adhesion molecules L-selectin, CD11b, and CD18 serve as neutrophil activation markers.

Activated tissue neutrophils contribute to further tissue injury by generation of oxygen free radicals, protease degranulation, promotion of

endothelial dysfunction, and recruitment of additional leukocytes (Liu et al. 2014). Negative feedback exists at several stages to control neutrophil influx to prevent neutrophil-mediated tissue damage, but under excessive neutrophil infiltration these mechanisms may fail (Borregaard 2010). Neutrophils play a central role in the development of local as well as systemic complications in SAP. In part through the production of oxygen free radicals, infiltrating neutrophils stimulate both acinar cell damage and pancreatitis-associated lung injury (Frossard et al. 1999, Gukovskaya et al. 2002). Indeed, depletion of neutrophils has been shown to attenuate experimental AP (Sandoval et al. 1996, Gukovskaya et al. 2002).

Neutrophils may contribute to host response also by expelling **neutrophil extracellular traps (NETs)** that can trap and kill invading bacteria (Brinkmann et al. 2004). NETs consist of smooth “threads”, composed of neutrophil deoxyribonucleic acid (DNA) and histones, covered with globular domains that contain granular proteins (Brinkmann et al. 2004). In vitro the NETs are expelled during specific neutrophilic cell death type, namely NETosis (Brinkmann and Zychlinsky 2007), but there is also data indicating that expelling NETs does not necessarily result in cell death (Yipp et al. 2012). The NETs are formed as a response to a variety of proinflammatory stimuli, such as TNF- α , IL-8, and lipopolysaccharide (LPS), and prevention of bacterial dissemination may be their main antibacterial function (Remijnsen et al. 2011, Leliefeld et al. 2016).

Excessive release of NETs causes cytotoxic effects, and growing evidence suggests that NETs have tissue-damaging properties (Liu et al. 2014, Leliefeld et al. 2016). Recently, NETs have been detected in the inflamed murine pancreas, and their possible role in recruitment of neutrophils and trypsinogen activation during experimental AP has been propounded (Korhonen et al. 2015, Merza et al. 2015). In another experimental study, neutrophils were observed to enter the lumen of biliopancreatic ducts under inflammatory conditions and form aggregated NETs, which then hampered secretory flow, thus driving focal pancreatitis (Leppkes et al. 2016).

Monocytes/Macrophages

Resident tissue macrophages are present virtually in all cell types, where they engulf dead cells, debris, and foreign material and orchestrate the inflammatory process. During inflammatory reaction circulating monocytes of bone marrow origin are recruited to the tissues and differentiate into macrophages (Varol et al. 2015). The migration through the endothelial wall is mediated by several adhesion molecules and is similar to that of neutrophils (Repo and Harlan 1999). Macrophages are the main source of pro- and anti-inflammatory cytokines, chemokines, and lipid mediators, and they recruit additional inflammatory cells to the site of inflammation (Xue et al. 2014). During inflammation macrophages are vivid phagocytes, and they also function as antigen-presenting cells, thus activating T cells of the adaptive immune system.

Lymphocytes

Lymphocytes include T and B cells and natural killer cells. While T and B cells are the primary cells of the adaptive immune system, natural killer cells participate in both innate and adaptive immune responses. Most of the circulating lymphocytes are T cells.

T cells mature in the thymus mainly to CD4 expressing T helper (Th) cells or CD8 expressing cytotoxic T cells. These antigen-naïve T cells circulate in the body and become activated in the lymph node during antigen presentation. T cell receptors of CD4+ cells engage peptides bearing major histocompatibility complex class II, such as human leukocyte antigen –antigen D related (HLA-DR), whereas CD8+ cells engage peptides bearing major histocompatibility complex class I. Depending on the type of antigen-presenting cell and the cytokine milieu at the site of antigen encounter, naïve CD4+ T cells differentiate into distinct populations (Th1, Th2, Th17, or Th9) that secrete a unique mixture of cytokines, although overlapping cytokine expression profiles are possible (Bonilla and Oettgen 2010).

Activated T cells migrate to the site of infection, where CD4+ T cells enhance both B and T cell response and CD8+ T cells eliminate pathogens by killing infected target cells (Bonilla and Oettgen 2010). During AP, CD4+ T cells have an important role in macrophage activation and they have also direct cytotoxicity effects on acinar cells (Demols et al. 2000). A small part of the circulating T cells is called regulatory T cells, which are actively involved in maintaining immune tolerance (Chatila 2005).

B cells reach maturity already within the bone marrow. After antigen encounter in lymphoid tissue, such as the spleen or lymph node, activated B cells develop into mature plasma cells and secrete immunoglobulins, the antigen-specific antibodies responsible for eliminating extracellular microorganisms (Delves and Roitt 2000).

Natural killer cells are a group of cytolytic lymphocytes that destroy infected and malignant cells. They also function as regulatory cells interacting with dendritic cells, macrophages, T cells, and endothelial cells (Delves and Roitt 2000).

Other cells

Dendritic cells are the most powerful antigen-presenting cells in the immune system, and they drive both innate and adaptive immune systems. Immature dendritic cells circulate in the body and recognize DAMPs through PRRs on their cell surface. After the binding to the receptor, immature dendritic cells become activated and migrate to nearby lymph nodes, where they process the antigen and present it to T cells. Dendritic cells have a crucial role in inflammation since they are the most powerful antigen-presenting cells and instructors of T cell response. A marked increase in the numbers of intrapancreatic dendritic cells has been shown in experimental AP, where they seem to have a dual role. They are capable of releasing proinflammatory

cytokines, but also protect the pancreas from severe injury. However, these mechanisms are not fully understood (Bedrosian et al. 2011).

Mast cells are a type of granulocyte found preferentially in the skin, mucosal surfaces, and around blood vessels. Upon activation, they proliferate at the site of inflammation and release vasoactive agents such as histamine, various inflammatory mediators including several leukocyte chemoattractants, and proteolytic enzymes from their intracellular granules. Mast cells are typically involved in allergic reaction and anaphylaxis. They have also capacity to phagocytize and kill bacteria, and they appear critical for the early neutrophil response to bacterial infection by secreting chemoattractants. Mast cells also contribute to adaptive immune responses through antigen presentation and release of immunoregulatory cytokines, thus influencing the development of specific T and B cell responses (Abraham and Arock 1998). During experimental AP activated mast cells have been shown to be involved in the development of endothelial barrier dysfunction in both the pancreas and extrapancreatic tissues, particularly in the lungs and colon, and contribute to the development of organ dysfunction (Dib et al. 2002).

Apart from controlling thrombosis and hemostasis, **platelets** are also involved in proinflammatory activities. They are activated by various inflammatory mediators, are a source of numerous chemokines, express various adhesion molecules, and release factors that can help to kill bacteria and infected cells (Semple and Freedman 2010). Platelets can also induce NET formation during bacterial infection (Clark et al. 2007), but not necessarily in sterile inflammation (Slaba et al. 2015).

2.2.3.2 Inflammatory mediators

Cytokines

Cytokines include interleukins, chemokines, interferons, colony-stimulating factors, and many growth factors. They are pleiotropic low-molecular-weight proteins that regulate host responses to infection, immune responses, inflammation, and trauma through cell-to-cell interactions, and are active in extremely small concentrations. There is also immense redundancy among cytokines, with many cytokines sharing similar biologic effects, and therefore, the traditional classification of cytokines as either proinflammatory or anti-inflammatory is somewhat artificial; many of the cytokines may have both effects depending e.g. on the time course of the immune response (Cavaillon 2001). Cytokines bind to specific cell surface receptors, and subsequent events of intracellular signaling then alter cell functions (Norman 1998, Dinarello 2000, Scheller et al. 2011). The systemic signs and symptoms of excess inflammatory cytokines are due to elevated cytokine levels, but also the receptors on potential target cells and the machinery to export the active protein are upregulated (Norman 1998, de Beaux et al. 1996).

TNF- α is a crucial first-line mediator of inflammation. The effects of TNF- α are transmitted through two different cell surface receptors, TNF- α receptor 1 and TNF- α receptor 2, which downregulate signaling cascades involving protein kinases and transcriptional factors, resulting in the induction of other cytokines (such as IL-6 and IL-8) and cell adhesion molecules (Malleo et al. 2007). TNF- α also intensifies oxidative stress and causes damage to the capillary endothelial cells and postcapillary venules that become procoagulant and proadhesive. Thus, TNF- α recruits and further activates neutrophils, resulting in further superoxide production and cell damage (Malleo et al. 2007). Finally, TNF- α orchestrates the spreading of local inflammatory reaction to the systemic illness.

IL-6 is elevated in most, if not all, inflammatory states. IL-6 induces all major acute-phase proteins in the liver, including C-reactive protein (CRP) (Castell et al. 1989). It is crucial to the resolution of acute neutrophil infiltration by inducing neutrophil apoptosis and inducing a switch from neutrophil to monocyte recruitment (Kaplanski et al. 2003, Chen et al. 2006, Scheller et al. 2011). IL-6 also affects many T cell activities; it induces T cell recruitment, controls the proliferation and survival of Th1 and Th2 lineage cells, is a key driver of the Th17 lineage, and can inhibit regulatory T cell functions (Hunter and Jones 2015). IL-6 stimulates target cells via either the *classic signaling* route, which mediates regenerative or anti-inflammatory activities, or through *trans-signaling*, which mediates proinflammatory responses (Scheller et al. 2011).

IL-8 is a chemokine that induces chemotaxis of leukocyte subsets (Adams and Lloyd 1997). It is primarily secreted by mononuclear phagocytes, but also by other cells, particularly endothelial cells, upon exposure to proinflammatory stimuli. IL-8 triggers neutrophil adhesion to the endothelium, directs migration into the tissue along the IL-8 gradient (recruitment of cells occurs towards an area of increased IL-8 concentration), and activates neutrophil effector mechanisms in the tissue (Adams and Lloyd 1997, Remick 2005). It is noteworthy that the biological activity of IL-8 is related more to the gradient of IL-8 rather than to the absolute IL-8 level. IL-8 may exert also anti-inflammatory effects if the gradient is in the wrong direction (away from the site of inflammation). Its unique feature in contrast to most inflammatory cytokines is that it may be produced early in the inflammatory response, but will persist for days or even weeks (Remick 2005).

Hepatocyte growth factor (HGF), originally identified as a mitogenic protein for rat hepatocytes (Nakamura et al. 1984, Russell et al. 1984), has multiple effects on tissue regeneration and is associated with proliferation, migration, and 3-D morphogenesis. The receptor for HGF is a Met tyrosine kinase. In mature tissues, HGF regulates cell survival by suppressing apoptosis, although it may also promote apoptosis of cells responsible for tissue fibrosis and induces expression of proteases (such as matrix metalloproteinases) involved in breakdown of the extracellular matrix scaffold (Nakamura et al. 2011). Recent studies have shown that HGF also regulates

the function of immune cells such as dendritic cells and a subset of regulatory T cells (Okunishi et al. 2005, Benkhoucha et al. 2010). Overall, the HGF-Met pathway prevents inflammation and fibrotic change in many tissues, and its tissue protective and/or regenerative effect has been demonstrated in several tissues, including kidney, lung, and gastrointestinal tissue. Clinical trials using recombinant human HGF protein or HGF genes are underway for numerous disease models in various tissues (Nakamura et al. 2011).

G-CSF is a crucial regulator of neutrophil production under both basal and stress conditions (Lieschke et al. 1994), and it also prolongs the survival of neutrophils and their precursors. For mature neutrophils, G-CSF enhances key functions such as superoxide production, phagocytosis, and bacteriocidal killing (Roberts 2005). Apart from neutrophils, G-CSF also influences dendritic and T cell function. In routine hospital protocol, G-CSF is used to increase the production of neutrophils in patients with chemotherapy-induced neutropenia (Roberts 2005).

Exogenic activators

LPS is a crucial component of an outer membrane that surrounds Gram-negative bacteria, such as *Escherichia coli* (*E. coli*), and mutants that are unable to form LPS are not viable. The outer membrane protects bacteria from toxic compounds (such as antibiotics) and mediates the physiological and pathophysiological interaction of bacteria with the host organism (Rietschel et al. 1994). Although most of the bacterial products can induce inflammation, LPS is one of the most powerful ones. The early step in cell activation by LPS is mediated by the LPS-binding protein to form LPS/LPS-binding protein complexes (Schumann et al. 1990), which are then recognized by PRRs such as CD14 (Wright et al. 1990) and TLR4 (Poltorak et al. 1998). Since CD14 lacks an intracellular domain, TLR4 is required for transmitting LPS signal from membrane-bound CD14 to the cytoplasm. Via soluble CD14 receptor, LPS may also activate CD14-negative cells such as endothelial cells (Heumann and Roger 2002).

E. coli is a Gram-negative coliform bacterium that belongs to the gut normal flora. It was first discovered by pediatrician Theodor Escherich (1885). There are several *E. coli* strains, some of which are pathogenic and some not. Pathogenic *E. coli* strains are responsible for infections of the gut, urinary tract, and lungs, among others. The virulence factors of different *E. coli* strains contain adhesins, iron acquisition systems, polysaccharide coats, and some have the ability to secrete toxins (Sannes et al. 2004, Vila et al. 2016). The complete genome of *E. coli* K-12 laboratory strain was published in 1997 (Blattner et al. 1997), and several hundred complete genomic sequences of *E. coli* are currently available.

Phorbol-12-myristate-13-acetate (PMA) is a phorbol ester commonly used in research to activate certain types of protein kinase C, and subsequently, MAP kinase pathways (Blumberg 1988, Seger and Krebs 1995).

2.2.3.3 Signaling pathways

Cell signaling is a process through which cells coordinate their actions according to the signals they recognize via cell surface receptors. Following the activation of a receptor, a cascade of events transports the signal ultimately to the nucleus, leading to an altered gene and protein synthesis with numerous impacts. The major inflammation-associated leukocyte signaling pathways include NF- κ B, ERK1/2 MAPK, and STATs.

2.2.3.3.1 NF- κ B

NF- κ B belongs to the Rel/NF- κ B family of transcription factors, including RelA, c-Rel, RelB, NF- κ B1 (p50 and its precursor protein p105), and NF- κ B2 (p52 and its precursor protein p100). They normally reside in the cytoplasm, where they are kept inactive by inhibitors of κ B (I κ B), the most important of which are I κ B α and I κ B β . Two major signaling pathways, the canonical and alternative NF- κ B pathways, exist (Rakonczay et al. 2008).

In the classical/canonical pathway (Figure 1), proinflammatory cytokines and PAMPs activate different receptors such as PRRs (including all TLRs), antigen receptors, and receptors for members of the TNF and IL-1 cytokine families. As a result, I κ B kinase complex is activated, and I κ Bs are rapidly phosphorylated at specific serine residues. This allows NF- κ B to translocate to the nucleus, where it binds DNA and activates gene transcription, such as TNF and IL-1 β , which amplify further NF- κ B activation (Bonizzi and Karin 2004, Rakonczay et al. 2008). The classical pathway is crucial in the innate immune responses. It encodes chemokines, cytokines, and adhesion molecules, such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, which recruit inflammatory cells to the site of inflammation.

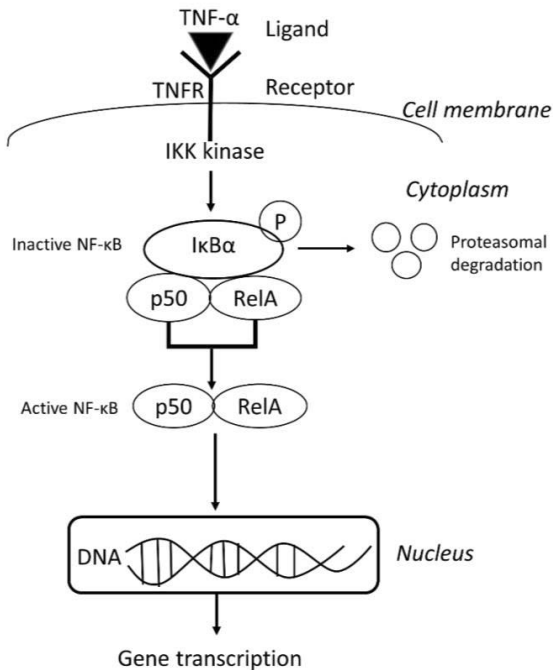


Figure 1 **NF-κB signaling (classical pathway).** Normally, NF-κB is inactive and resides in the cytoplasm. When cells are stimulated by an activating signal, such as TNF-α, IκB kinase rapidly phosphorylates inhibitors of κB and targets them for proteasomal degradation. When IκB degrades, nuclear translocation signals of NF-κB are unmasked, and NF-κB is able to translocate into the nucleus, where it binds to its cognate DNA sequence and induces the transcription of its target genes. *Abbreviations:* IκBα, Inhibitor of κB; TNF, Tumor necrosis factor; TNFR, Tumor necrosis factor receptor

NF-κB activation can also occur independently of IκB phosphorylation or degradation; this is known as the alternative or non-canonical pathway. During the inflammatory response the alternative NF-κB pathway is suggested to have a role in lymphoid organ development and adaptive immunity (Vallabhapurapu and Karin 2009).

2.2.3.3.2 MAPK

The MAPK signaling pathway promotes cellular processes, such as proliferation, differentiation, and development, and dysregulation of this pathway is common in cancer. ERK1 and ERK2, collectively called ERK1/2 due to their high degree of similarity, are among the 14 MAPKs described in mammals. Each of these signaling cascades consists of several tiers of different protein kinases that sequentially activate each other by phosphorylation allowing for rapid and regulated transmission of the original initiating signal.

The core cascade is usually composed of three tiers: mitogen-activated protein kinase kinase kinase (MAP3K), MAP2K, and MAPK (Rubinfeld and Seger 2005). The upstream activation of MAP3Ks is complex, and different MAP3Ks can have varied activation mechanisms. For example, tumor progression locus 2 is a MAP3K for the ERK1/2 pathway (Arthur and Ley 2013). During the innate immune response MAPK activation has been mostly studied in macrophages and dendritic cells in the context of TLR agonists (Figure 2).

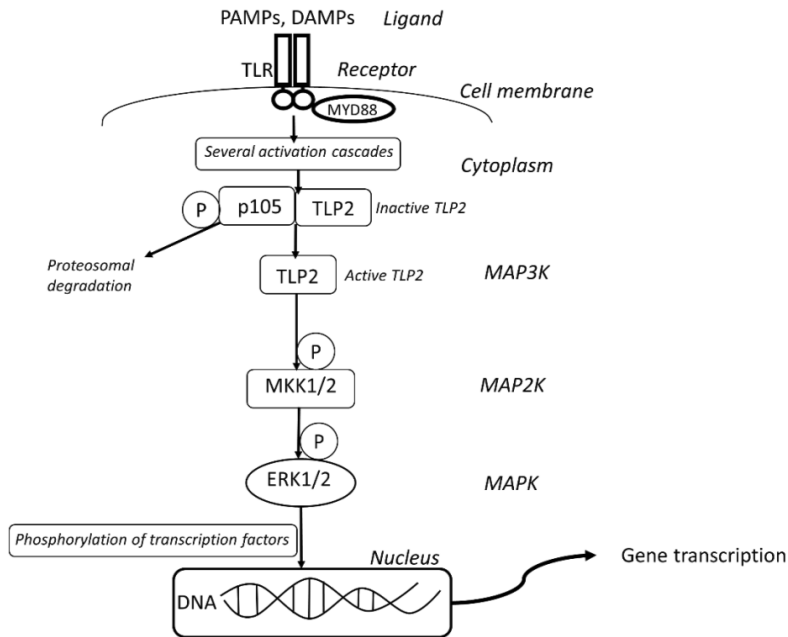


Figure 2 *ERK1/2 signaling following activation of Toll-like receptor.* Following activation of Toll-like receptor (TLR), myeloid differentiation primary-response protein 88 (MYD88) is recruited to the intracellular domain of the receptor, thus initiating a cascade of events that eventually leads to the activation of tumor progression locus 2 (TLP2). Active TLP2 phosphorylates MAPK kinase 1 and 2 (MKK1/2), which subsequently leads to the phosphorylation of ERK1/2, further phosphorylating transcription factors that control gene expression in the nucleus. *Abbreviations:* DAMP, Damage-associated molecular pattern; MAPK, Mitogen-activated protein kinase; MAP2K, Mitogen-activated protein kinase kinase; MAP3K, Mitogen-activated protein kinase kinase kinase; MKK1/2, Mitogen-activated protein kinase 1 and 2; MYD88, Myeloid differentiation primary-response protein 88; PAMP, Pattern-associated molecular pattern; TLP2, Tumor progression locus 2; TLR, Toll-like receptor.

2.2.3.3.3 STATs

The Janus kinase (JAK)-STAT signaling pathway is employed in the signaling of many cytokines. Cytokine receptors lack, in general, intrinsic tyrosine kinase activity, therefore requiring an association with receptor-associated kinases (i.e. JAKs) in order to propagate a phosphorylation cascade. Cytokine receptor phosphorylation allows binding of STATs, after which phosphorylation of STATs by JAKs ensues. Phosphorylation leads to STAT homo- and heterodimerization. STAT dimers are rapidly transported from the cytoplasm to the nucleus and bind to DNA in order to alter transcription (Aaronson and Horvath 2002, Scott et al. 2002).

There are seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) and four JAKs (JAK1, JAK2, JAK3, and TYK2) in mammals. STAT1, STAT2, STAT3, STAT5, and STAT6 are present in various tissues. In an experimental mouse model of AP, these STATs were also present in pancreatic tissue, where acinar cells were the main source of STAT1, STAT2, STAT3, and STAT5, and STAT6 originated from non-acinar cells (Gallmeier et al. 2005). Regulation of JAK/STAT pathways is crucial in immune response, and the pathway is regulated by a number of intrinsic and environmental stimuli. Negative regulation pathways include receptor degradation and dephosphorylation of activated STAT dimers and suppressors of cytokine signaling proteins that inactivate JAKs (Aaronson and Horvath 2002, Scott et al. 2002).

STAT1 forms part of a major signaling pathway for interferon gamma, a proinflammatory cytokine that increases Th1 differentiation and cell-mediated immune responses and is crucial for the activation of macrophages and monocytes (Kim and Maniatis 1996, Scott et al. 2002). STAT1-deficient mice have been shown to possess a complete lack of responsiveness to interferon gamma and to be highly sensitive to infection by microbial pathogens and viruses (Meraz et al. 1996).

The **STAT3** pathway (Figure 3) was first discovered as a mediator of acute-phase response in the liver induced by IL-6, IL-1 β , and TNF- α (Akira et al. 1994). Later, it has been shown to be activated also by various other cytokines such as G-CSF, epidermal growth factor, leptin, and IL-10 (Takeda et al. 1999). STAT3-deficient knock-out mice die early in the fetal stage, but tissue-specific targeting of STAT3 causes only distinctive abnormalities. For example, STAT3 deficiency in T cells impaired IL-2 and IL-6-induced T cell proliferation (Takeda et al. 1998), and in macrophages and neutrophils STAT3 deficiency led to impaired IL-10 responsiveness (Matsukawa et al. 2003).

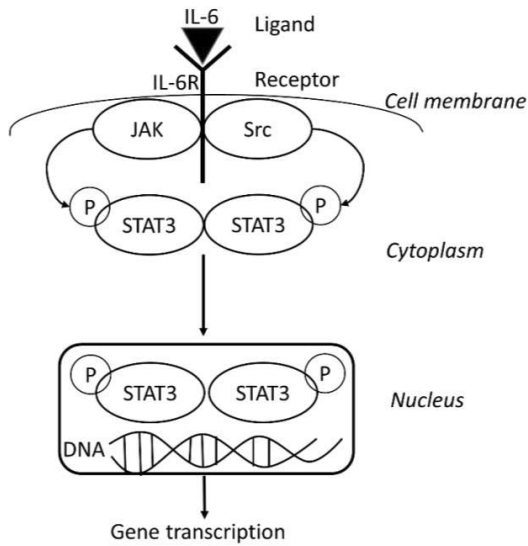


Figure 3 **STAT3 signaling.** STAT3 signaling pathway is activated when a ligand, such as IL-6, binds to its receptor. This leads to the recruitment and activation of the JAK family of proteins, which in turn recruits and phosphorylates latent STAT3 in the cytoplasm. STAT3 can also be directly phosphorylated by non-tyrosine kinase receptors (Src). Phosphorylated STAT3 homodimerizes and translocates to the nucleus, where it regulates gene expression. *Abbreviations:* IL, Interleukin; IL6R, IL-6 receptor; JAK, Janus kinase; Src, Src-kinase; STAT, Signal transducer and activator of transcription.

2.2.4 ACINAR CELL DEATH

Along with the host's inflammatory reaction, cell death modality is one of the key factors determining the course and prognosis of AP.

2.2.4.1 Apoptosis

Apoptosis is a tightly regulated form of cell death, morphologically characterized by cell shrinkage, integral cell membrane, nuclear condensation, and formation of apoptotic bodies (Kerr et al. 1972). Extracellular or intracellular stress signals initiate apoptosis, mainly through caspase-dependent pathways (Thornberry and Lazebnik 1998, Galluzzi et al. 2012). Apoptotic bodies are recognized by phagocytes and engulfed before they leak their contents into the extracellular space. (Taylor et al. 2008). Developmentally programmed apoptotic cell death is crucial for life and is considered immunologically silent. However, pathological apoptotic cell death indicates tissue injury and should be detected by the immune system. Indeed, recent studies suggest that in certain situations apoptotic cells can be proinflammatory through the release of a limited amount of DAMPs, or

apoptotic cells themselves can produce cytokines and chemokines, thus actively engaging the immune system (Cullen et al. 2013, Kearney et al. 2013, Wickman et al. 2013). On the other hand, apoptotic cell death has been shown to suppress inflammation through cell surface changes, which induce the generation of anti-inflammatory mediators (Voll et al. 1997). In experimental AP studies, apoptosis has been demonstrated to be the major form of cell death in mild AP (Kaiser et al. 1995).

2.2.4.2 Regulated necrosis

Necrotic cells exhibit translucent cytoplasm, swelling of organelles, and disruption of the plasma membrane with the release of endogenous molecules (i.e. DAMPS) that directly trigger a proinflammatory response (Kaczmarek et al. 2013). In the past, necrosis was thought to be an unscheduled and unregulated form of cell death induced by overwhelming external stress, but growing evidence indicates that, at least in part, necrotic cell death is finely regulated by a set of intracellular signal transduction pathways (Golstein and Kroemer 2007). Regulated necrosis can be further characterized with regard to its dependence on specific signaling modules into necroptosis, parthanatos, ferroptosis, oxytosis, mitochondrial permeability transition-dependent necrosis, pyroptosis, and pyronecrosis, and cell death is associated with the release of (neutrophil) extracellular traps, which is described as NETosis (ETosis) (Pasparakis and Vandenabeele 2015).

The best-characterized form of regulated cell necrosis, which has been detected in experimental AP, is **necroptosis** (Zhang et al. 2009, Sun et al. 2012, Wu et al. 2013, Ma et al. 2015, Louhimo et al. 2016). By definition, necroptosis can be further characterized with regard to its dependence on specific signaling modules (receptor-interacting protein 1 or receptor-interacting protein 3 dependent) (Galluzzi et al. 2012). Morphologically, necroptosis is similar to necrosis, and necroptosis is also thought to result in the release of DAMPs into the extracellular space. For this reason, necroptosis is currently considered to be a highly inflammatory mode of cell death (Davidovich et al. 2014). Since several of the upstream signaling elements of apoptosis and necroptosis are shared, overlapping mechanisms between different cell death types exist (Linkermann and Green 2014). Recent preliminary studies have also shown that inhibition of necroptosis would be beneficial in experimental AP (Zhang et al. 2009, Sun et al. 2012, Wu et al. 2013, Ma et al. 2015), even after the onset of AP (Louhimo et al. 2016). Also blocking necroptosis in TNF-induced systemic inflammatory response syndrome (SIRS) protected mice against lethal SIRS (Duprez et al. 2011).

2.2.4.3 Nucleosomes

The nucleosome is an example of a nuclear DAMP. Besides nucleosomes, other DAMPs include nucleosome components (histones and DNA), high-mobility group box 1 (HMGB1), s100 proteins, heat shock proteins, hyaluronic acid, uric acid, adenosine triphosphate, and ribonucleic acid (Tang et al. 2012, Kang et al. 2014a). The nucleosome is a basic unit of nuclear chromatin, and it is composed of a central core protein formed by an octamer of the double-represented histone and 147 pairs of double-stranded DNA. Single nucleosomes are connected by so-called linker DNA, and a further histone is located at these linking sites outside the nucleosomes, stabilizing the chain in its tertiary structure. The nucleosome structural organization plays an essential role in regulating gene transcription and facilitates efficient higher-order chromatin compaction (Oudet et al. 1975, Luger 2003).

Upon physiological cellular damage, nucleosomes are released into the extracellular space, where they are engulfed by macrophages and neighboring cells (Bell and Morrison 1991). Small levels of circulating nucleosomes can be found in healthy persons, but enhanced cell death (apoptosis, regulated necrosis, NETosis) leads to impaired elimination systems, and thus, higher circulating nucleosome levels are found in various pathologic conditions (Holdenrieder and Stieber 2009). Elevated nucleosome levels have also been found in experimental AP (Kang et al. 2014b). Moreover, the nucleosome components, DNA and histone, serve as DAMPs; elevated levels of circulating DNA have been found to be associated with the severity of human AP (Gornik et al. 2009, Kocsis et al. 2009, Gornik et al. 2011), and elevated histone levels with the severity of experimental AP (Ou et al. 2015).

The origin of circulating nucleosomes, but also circulating histones and cell-free DNA, seems to be diverse, including dying non-myeloid cells through apoptotic and necrotic cell death, but also dying activated neutrophils (a cell death form called “NETosis”) (reviewed in Marsman et al. 2016). Of note, nucleosomes, histones, and cell-free DNA seem to have differences in immunostimulation e.g. the cytotoxic effects ascribed to histones do not appear to apply to nucleosomes (Gauthier et al. 1996, Xu et al. 2009, Marsman et al. 2016). Nucleosomes contribute to immune reaction by, for instance, inducing neutrophil and dendritic cell activation and subsequent proinflammatory cytokine production (Decker et al. 2005, Lindau et al. 2011,). Nucleosomes may also form complexes with another potent nuclear DAMP, namely HMGB1, which in turn activates macrophages to produce cytokines (Urbonaviciute et al. 2008). Cell-surface proteoglycans have been found to be involved in the binding of nucleosomes to cell surfaces (Watson et al. 1999). In addition, the existence of a nucleosome-specific receptor has been proposed, but has not yet been identified (Marsman et al. 2016).

2.2.5 LOCAL AND SYSTEMIC INFLAMMATORY RESPONSE

Proinflammatory response

The severity of AP attack is determined by the extent of inflammatory reaction and the host's response to it, not by the amount of pancreatic damage. According to the current theory, local activation of NF- κ B in acinar cells, and acinar cell death caused by premature trypsinogen activation are two early parallel events after the onset of AP that induce local inflammation in the pancreas (Gukovsky et al. 1998, Rakonczay et al. 2008). The NF- κ B signaling pathway mediates the production of proinflammatory cytokines, such as TNF- α and IL-1 β , which then activate other signaling pathways, such as STAT1, STAT3, and ERK1/2 MAPK, in the pancreas (Dabrowski et al. 1996, Gallmeier et al. 2005). Acinar cell death leads to the release of immunogenic DAMPs outside the acinar cells that activate downstream signaling pathways, resulting in increased transcription of inflammatory genes and subsequent production of proinflammatory cytokines (Chen and Nunez 2010).

Release of proinflammatory cytokines and chemokines activates the endothelium, and circulating inflammatory cells, first neutrophils and then monocytes/macrophages and lymphocytes, are recruited into the pancreas. Sequestered inflammatory cells, especially macrophages, in turn produce more cytokines and recruit additional leukocytes into the pancreas, which is later followed by leukocyte recruitment into distant organs such as the lung (McKay et al. 1996). Proinflammatory cytokines also activate tissue resident macrophages in remote organs (such as the peritoneum, liver, and lungs), which then produce proinflammatory cytokines, thus contributing to the systemic progression of AP (Shrivastava and Bhatia 2010). The activated immune system tries to resolve the local inflammation, generally succeeding, but if it fails AP may rapidly progress to systemic illness.

In SAP, the severity of systemic inflammatory response is comparable to that seen in bacterial sepsis, with similar clinical symptoms and pattern of released inflammatory mediators (Deitch 1992, Wilson et al. 1998). The vicious circle that promotes the amplification of a local response into a systemic response (Figure 4) involves the extrapancreatic activation of NF- κ B and other signaling pathways, systemic production of proinflammatory cytokines, and excessive neutrophil infiltration in remote organs (Rakonczay et al. 2008, Kylänpää et al. 2012). Excessive and uncontrolled neutrophil infiltration leads to the accumulation of toxic neutrophil products that induce cell death and damage to vascular endothelial cells. Subsequently, endothelial permeability is increased, resulting in the accumulation of tissue fluid and edema (Kylänpää et al. 2012). Together with microvascular disturbances (e.g. vasoconstriction, inadequate perfusion, and increased blood viscosity), excessive tissue fluid leads to a lack of oxygen, which results in dysfunction and injury of end organs (Menger et al. 2001). Although AP is a sterile disorder in the early phase (Beger et al. 1986), increased gut permeability may allow

bacterial translocation and endotoxins to the circulation. This may cause infection of the necrotic pancreas, and even sepsis (Capurso et al. 2012).

Along with the activation of inflammatory pathways, the pathogenesis of OD consists of major modifications to non-immunological pathways, such as those of cardio-vascular, neuronal, autonomic, hormonal, bioenergetic, metabolic, and coagulation systems, all of which have prognostic significance (Singer et al. 2004).

Compensatory anti-inflammatory response

Early and simultaneously with the proinflammatory reaction, the anti-inflammatory response occurs to downregulate the process, which is documented by the presence of both pro- and anti-inflammatory cytokines in the circulation (Makhija and Kingsnorth 2002, Mentula et al. 2004). This phenomenon has been called compensatory anti-inflammatory response syndrome (CARS) (Bone 1996). Although the exact mechanisms are still poorly understood, the current theory suggests that an excessive anti-inflammatory response may cause immunosuppression, which, on a cellular level, is present already in the early phase of SAP and may be linked to increased susceptibility to subsequent infections (Figure 5) (Li et al. 2013, Pan et al. 2017).

The signs of immunological impairment include defects in leukocyte signaling (Oiva et al. 2010a, 2010b, 2013), reduced monocyte HLA-DR expression (Kylänpää-Bäck et al. 2001a, Mentula et al. 2003), and lymphocyte dysfunctions, shown as delayed hypersensitivity in skin testing (Garcia-Sabrido et al. 1989), reduced circulating lymphocyte count (Christophi et al. 1985, Takeyama et al. 2000), and impaired activity of CD4+ T cells (Curley et al. 1993, Pezzilli et al. 1994). Despite the cellular signs of immunosuppression, clinically, a hyperinflammatory phase predominates with shock, fever, and hyper-metabolism.

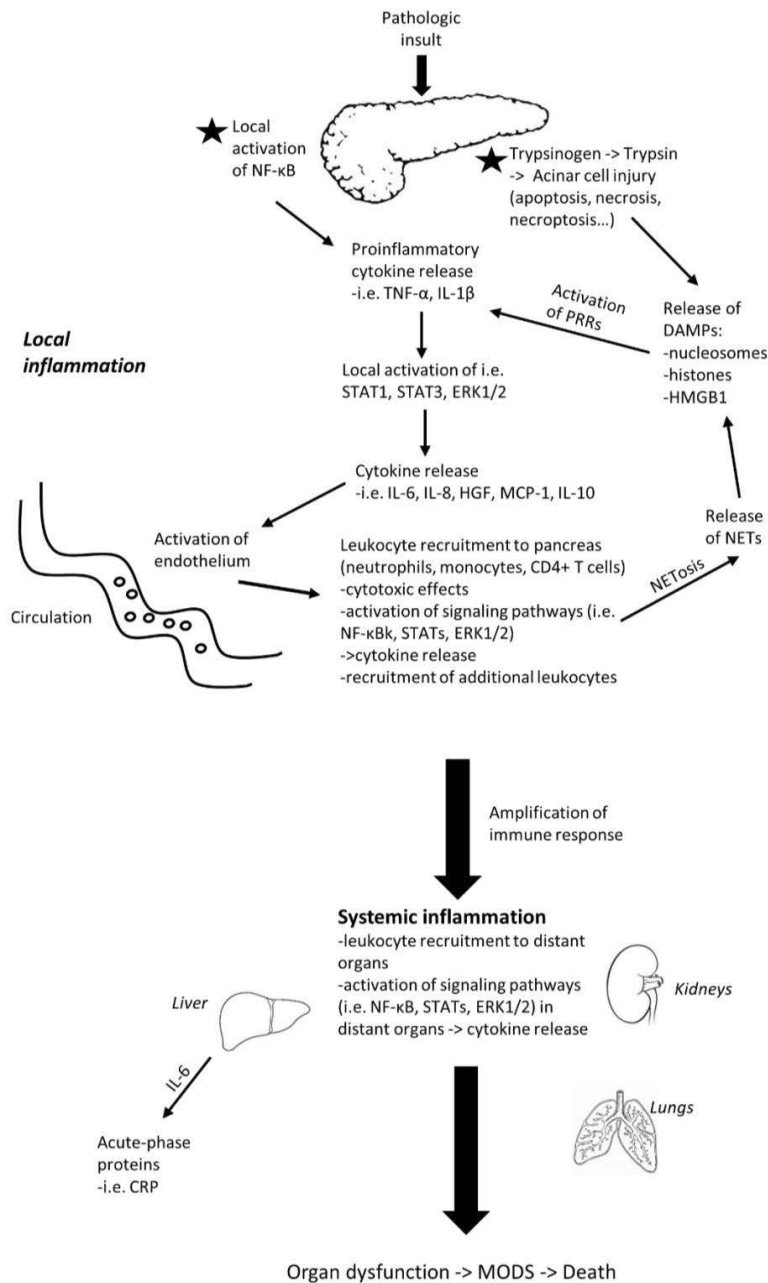


Figure 4 *Spreading of local inflammatory reaction into systemic response in acute pancreatitis (simplified).* Abbreviations: CRP, C-reactive protein; DAMP, Damage-associated molecular pattern; ERK, Extracellular signal-regulated kinase; HGF, Hepatocyte growth factor; HMGB1, High mobility group box 1 protein; IL, Interleukin; MCP, Monocyte chemoattractant peptide; MODS, Multiple organ dysfunction syndrome; NET, Nuclear extracellular trap; NF-κB, Nuclear factor kappa B; PRR, Pattern recognition receptor; STAT, Signal transducer and activator of transcription; TNF, Tumor necrosis factor.

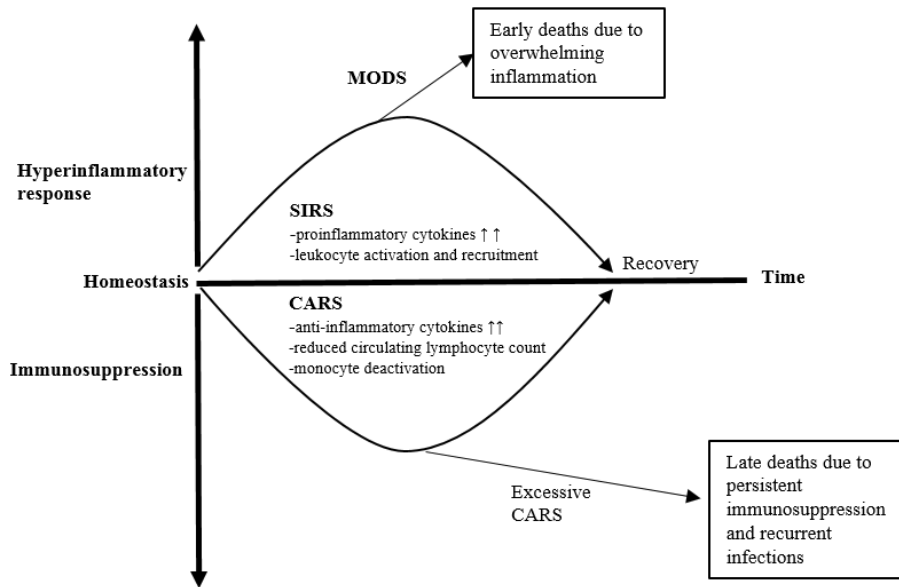


Figure 5 *Course of systemic inflammatory reaction* (modified from Hotchkiss et al. 2013).
Abbreviations: CARS, Compensatory anti-inflammatory response; MODS, Multiorgan dysfunction syndrome; SIRS, Systemic inflammatory response syndrome.

2.3 DIAGNOSIS OF ACUTE PANCREATITIS

2.3.1 MAIN DIAGNOSTIC CRITERIA

The diagnosis of AP requires abdominal pain (acute onset of persistent, severe, epigastric pain, often radiating to the back) together with serum lipase or amylase activity at least three times greater than the upper limit of normal, and/or characteristic findings of AP on abdominal imaging. CECT and magnetic resonance imaging (MRI) should be reserved for patients in whom the diagnosis is unclear or who fail to improve clinically within the first 48-72 hours or to evaluate complications (Tenner et al. 2013).

2.3.2 CLINICAL SYMPTOMS AND SIGNS

The pain usually starts acutely in the upper abdomen and is often described as a belt-like pain radiating to the back. The pain worsens rapidly and later involves the whole abdomen. The onset of AP is defined as the time of onset of abdominal pain, and the time interval between onset of pain and first

admission to hospital should be noted (Banks et al. 2013). Other gastrointestinal symptoms include nausea and vomiting. Very rare but severe symptoms are hematemesis and melena (Lankisch et al. 2015). In the physical examination, most patients experience general palpation as painful. Peritoneal irritation is often present in a severe attack. Cutaneous signs, i.e. brownish-green discoloration in the umbilical region (Cullen's sign), in the flanks (Grey Turner's sign), or in the upper thigh (Fox's sign), indicate SAP and are associated with increased risk of mortality, but they are seen only rarely. In a prospective study of 425 patients with AP, skin manifestations were present in 1.2% of all AP patients and in 8% of SAP patients (Lankisch et al. 2009).

2.3.3 LABORATORY EXAMS

Amylase (preferably pancreas-specific) and lipase activities are used for early diagnosis of AP, but their levels are not associated with disease severity (Lankisch et al. 1999). Both amylase and lipase levels increase early after onset of AP, but amylase is quicker to return to normal. If there is a delay of 2-3 days before seeking treatment, amylase may be only slightly elevated or even normal. It has also been shown that in patients with alcohol-induced AP the amylase levels are lower than in other etiologic groups (Lankisch et al. 1999). Besides AP, amylase, including also pancreas-specific amylase, and lipase can be elevated in numerous other intra-abdominal diseases such as perforating duodenal ulcer, cholecystitis, ileus, diabetic ketoacidosis, appendicitis, kidney failure, some neoplasms, and macroamylasemia (Pieper-Bigelow et al. 1990). Alternative methods for early diagnosis of AP exist, but have limited availability. For example, urinary trypsinogen-2 dipstick is a rapid and non-invasive method with an adequate diagnostic accuracy (Hedström et al. 1996, Kemppainen et al. 1997, Kylänpää-Bäck et al. 2000, Chang et al. 2012).

To assess the severity of AP on admission, the following laboratory parameters are recommended: complete blood count, CRP, concentrations of electrolytes, creatinine, liver transaminases, alkaline phosphatase, blood glucose, coagulation status, and total albumin. If oxygen saturation is less than 95% or the patient is tachypneic, arterial blood gas analysis is recommended (Lankisch et al. 2015).

2.3.4 IMAGING

CECT is the gold standard for diagnostic imaging, but early CT scan is recommended only when there is clinical doubt about the AP diagnosis, and other life-threatening disorders have to be excluded; it should not be used solely for severity assessment (Banks et al. 2013, Lankisch et al. 2015). The reasons for this are as follows: it takes several days before necrosis can be clearly defined; the morphologic changes are not directly proportional to the

severity of OD; in the early phase of AP, no treatments are required for fluid collections or pancreatic necrosis; and the predictive accuracy of CT scoring systems for determining the severity of AP is similar to that of clinical scoring systems (Bollen et al. 2012, Banks et al. 2013). Impaired kidney function and allergy are contraindications for the use of contrast medium.

Non-enhanced MRI is comparable to CECT in the early assessment of severity of AP (Stimac et al. 2007), but MRI is better than CECT to distinguish solid masses from liquid component, and thus, may help in determining necrosis in the fluid collection (Banks et al. 2013). Transabdominal ultrasonography is the first-line imaging modality for detecting gallbladder stones, and magnetic resonance cholangiopancreatography (MRCP) for common bile duct stones (Giljaca et al. 2015, Gurusamy et al. 2015). A simple chest x-ray can show pleural effusions and pulmonary infiltrates.

2.4 CLASSIFICATION OF ACUTE PANCREATITIS

The Atlanta classification based on the Atlanta Symposium in 1992 is the standard classification system for AP severity, distinguishing mild and severe AP (Bradley 1993). This classification was recently revised, now separating three different groups: mild, moderately severe, and severe (Table 2) (Banks et al. 2013). Patients with mild AP recover uneventfully and show no signs of organ dysfunction or exacerbation of chronic illness or any local complications on CECT. Patients with moderately severe AP develop local morphologic complications seen on CECT and/or exacerbation of chronic illness and/or transient OD that resolves within 48 hours. Patients with SAP suffer from persistent OD that lasts more than 48 hours. The presence of OD is assessed according to the Modified Marshall Score (MMS), where 2 points or more in any of the evaluated organ systems indicate OD (Marshall et al. 1995, Banks et al. 2013).

Table 2. *Revised Atlanta Classification (Banks et al. 2013).*

Clinical/radiological findings	Severity of acute pancreatitis		
	Mild	Moderately severe	Severe
Uneventful recovery	+	-	-
Local complication on CT	-	-/+	-/+
Exacerbation of chronic illness	-	-/+	-/+
Organ dysfunction	-	-/transient ^a	persistent ^b (always present)

Abbreviations: CT, Computed tomography

^aOrgan dysfunction resolves within 48 hours

^bOrgan dysfunction persists for more than 48 hours

2.4.1 MORPHOLOGY

According to the Revised Atlanta Classification (Banks et al. 2013), AP can be subdivided into two groups based on morphologic findings in CECT: (1) edematous pancreatitis with acute inflammation of the pancreatic parenchyma and peripancreatic tissues, but without recognizable tissue necrosis, and (2) necrotizing pancreatitis with acute inflammation associated with pancreatic parenchymal necrosis and/or peripancreatic necrosis. Different types of local complications occur upon edematous and necrotizing pancreatitis. In edematous pancreatitis a homogenous fluid collection without definable walls and adjacent to pancreas is called acute peripancreatic fluid collection. If acute peripancreatic fluid collection develops a well-defined encapsulated wall around itself, it is called a pancreatic pseudocyst. This takes usually more than four weeks. Necrotic collections associated with necrotizing pancreatitis include acute necrotic collection, which may eventually (after four weeks) mature into walled-off necrosis surrounded by a well-defined wall. Acute necrotic collection and walled-off necrosis may be intrapancreatic and/or extrapancreatic. Correct classification of a collection is required, since the treatment modalities may differ (Banks et al. 2013).

2.4.2 EARLY AND LATE PHASE CLASSIFICATION

During the first week of hospitalization (early phase) systemic complications define the treatment strategy. Systemic spread of the cytokines manifests clinically as SIRS. SIRS is present if at least two of the following features exist: temperature $<36^{\circ}\text{C}$ or $>38^{\circ}\text{C}$; pulse >90 per minute; respiratory rate >20 breaths per minute; and white cell count <4 or $>12 \times 10^9/\text{l}$ (American College of Chest Physicians 1992). Early SIRS and OD are rather common findings on admission to hospital, but often the host's response to inflammation adapts to treatment, and in many patients the systemic signs of inflammation resolve within the first 48 hours of admission (Buter et al. 2002, Mofidi et al. 2006). About half of the SAP patients present with OD or develop it during the first 24 hours (Johnson et al. 2001, Mentula et al. 2003). Apart from AP-associated OD, a pre-existing comorbidity may also be exacerbated by AP. If this happens, AP is classified as moderately severe (Banks et al. 2013). Persistent OD that does not resolve within 48 hours is the defining feature of SAP.

The later phase overlaps with the early phase, but generally starts after the second week and may last for weeks or months. The late phase is characterized by persistence of systemic signs of inflammation or the presence of local complications. During this phase the local complications gradually become encapsulated and may require treatment. Therefore, defining the morphologic characteristic of local complications by radiologic imaging becomes important (Banks et al. 2013). Even though the cellular signs of CARS or

immunosuppression may be present already on admission (Kylänpää-Bäck et al. 2001a, Mentula et al. 2003), the infectious clinical complications usually develop in the later phase (Beger et al. 1986) as a result of bacterial translocation from gut lumen into the circulation (Capurso et al. 2012).

Mortality in SAP is largely associated with the development of MODS and infected pancreatic necrosis (Halonen et al. 2002, Johnson and Abu-Hilal 2004, Petrov et al. 2010). MODS generally follows a predictable course, beginning with the lungs and followed by hepatic, intestinal, and renal dysfunction. Hematologic and myocardial failure usually occur later, whereas central nervous system dysfunction may occur early or late. Disorders of the immunologic system occur early at the cellular level (Kylänpää et al. 2005). Over the last years, the mortality in AP has declined; recently, an overall mortality rate of only 0.9% was reported in the United States (Krishna et al. 2017). Recent studies show the mortality rates in relation to the severity of AP as 0% in mild AP, 0-3% in moderately severe AP, and 15-59% in SAP (Nawaz et al. 2013, Zubia-Olaskoaga et al. 2016). Development of MODS substantially increases the risk of death. While in the presence of a single OD the mortality rate is less than 10%, in the presence of MODS it rises to 35-50% (Pandol et al. 2007). Mortality is biphasic. Half of the deaths occur within the early phase due to early MODS (McKay and Imrie 2004) and the other half in the late phase most often due to septic complications. Also in late mortality early MODS is a predisposing risk factor (Isenmann et al. 2001, Buter et al. 2002).

2.5 TREATMENT OF ACUTE PANCREATITIS

2.5.1 CONSERVATIVE TREATMENT

Fluid therapy

The early treatment of AP includes early fluid resuscitation, supplemental oxygen, analgesia, and nutritional support (Lankisch et al. 2015). Although fluid therapy is considered a cornerstone of early treatment, evidence of its positive effects is based on animal models showing that AP compromises splanchnic perfusion and pancreatic microcirculation (Gardner et al. 2008). A recent systematic review claimed the current evidence of fluid therapy to be paltry and of poor quality, and several important questions, such as the type of fluid, the rate of administration, and how fluid therapy should be guided, remain to be answered (Haydock et al. 2013). Recent guidelines (Tenner et al. 2013, Working Group IAP/APA Acute Pancreatitis Guidelines 2013) suggest that early aggressive fluid therapy is most beneficial within the first 12-24 hours, preferably with Lactate Ringer's solution. It is crucial to assess the response to fluid therapy every 6 hours for at least 24-48 hours.

Nutrition

Among all AP patients, early enteral feeding is recommended, and parenteral nutrition is administered only in rare cases when enteral feeding is not tolerated. Provision of enteral feeding has been shown to downregulate systemic immune responses, reduce oxidative stress, reduce the rate of infections, and improve patient outcome compared with total parenteral nutrition (McClave and Heyland 2009, Al-Omran et al. 2010). In mild AP without severe pain, nausea, or vomiting, oral feeding may be restarted without delay. In SAP, enteral tube feeding through nasojejunal or nasogastric tube is recommended for patients who do not tolerate oral feeding by day 3 to day 5 (Forsmark et al. 2016).

Antibiotics

Although much debated, the current guidelines state that antibiotic prophylaxis is not recommended in AP, unless infection is suspected or has been confirmed (Tenner et al. 2013, Working Group IAP/APA Acute Pancreatitis Guidelines 2013). If infected pancreatic necrosis is diagnosed or strongly suspected, use of broad-spectrum antibiotics covering both Gram-positive and Gram-negative bacteria of the normal intestinal flora, which is a common source of infection (Beger et al. 1986), is advised.

2.5.2 INVASIVE TREATMENT

Abdominal compartment syndrome

Normal intra-abdominal pressure is < 12 mmHg, whereas intra-abdominal pressure of 12-20 mmHg is defined as intra-abdominal hypertension, and intra-abdominal pressure > 20 mmHg with new onset of OD as abdominal compartment syndrome. In SAP, different studies show the incidence for intra-abdominal hypertension to be 60-80% and that for abdominal compartment syndrome 25-50% (De Waele and Leppäniemi 2009). First-line treatment is conservative decompression with nasogastric drainage and rectal tubes, if necessary, along with prokinetics, depletion of volume overload, and drainage of ascites. Also adequate analgesia and sedation to decrease abdominal muscle tone should be provided. Only if these fail and intra-abdominal pressure is constantly > 25 mmHg with new onset of OD, surgical decompression should be performed (Mentula et al. 2010, Working Group IAP/APA Acute Pancreatitis Guidelines 2013).

Infected necrosis

Indications for radiological, endoscopic, or surgical intervention in necrotizing pancreatitis are infected necrosis with clinical deterioration and, or in the absence of documented infected necrosis, ongoing OD for several weeks after the onset of AP. There is a consensus that endoscopic or surgical intervention,

if necessary, should be delayed until 4 weeks after onset of AP to allow the collection to mature and become “walled-off” (Working Group IAP/APA Acute Pancreatitis Guidelines 2013). In addition to open necrosectomy, mini-invasive necrosectomy techniques have emerged, such as endoscopic transgastric necrosectomy (Seifert et al. 2009), and a “step-up approach” that comprises placement of percutaneous catheters, followed by minimally invasive necrosectomy if necessary (van Santvoort et al. 2010).

Other reasons

Besides infected necrosis, ongoing gastric outlet or intestinal or biliary obstruction due to mass effect, forming large uninfected walled-off necrosis, may require intervention, preferably 4-8 weeks after onset of AP. Acute bleeding or bowel ischemia are acute situations that require emergency laparotomy (Working Group IAP/APA Acute Pancreatitis Guidelines 2013). Pseudoaneurysms are rare, but potentially serious complications of AP. They are treated through mesenteric angiography with transcatheter arterial embolization (Lankisch et al. 2015). In mild biliary pancreatitis, early cholecystectomy during the same hospital admission is advised (da Costa et al. 2015). In necrotizing AP, it is recommended to delay cholecystectomy for 6 weeks, until active inflammation subsides and fluid collections resolve or stabilize (Working Group IAP/APA Acute Pancreatitis Guidelines 2013). If a patient is unfit for surgery, endoscopic sphincterotomy is recommended (Lankisch et al. 2015). Early ERCP is recommended only in severe biliary pancreatitis and subsequent cholangitis due to biliary obstruction (Tse and Yuan 2012).

2.6 PREDICTING THE SEVERITY OF ACUTE PANCREATITIS

Besides making an accurate diagnosis of AP at the emergency department and starting optimal care, it is crucial to assess the severity of AP to determine which patients will have SAP, requiring earlier triage to intermediate care or ICU and earlier initiation of effective therapy. On the other hand, the limited ICU resources should be allocated wisely (Lilja et al. 2008). Clinical assessment has been shown to correctly identify only one-third of SAP patients on admission (Wilson et al. 1990). Therefore, several clinical and laboratory markers and various scoring systems have been evaluated as optimal predictive markers for the first 48 hours.

2.6.1 CLINICAL FACTORS AND SCORING SYSTEMS

Clinical factors that increase the risk of complications or death include age \geq 60 years, comorbidity, alcohol use, smoking, body mass index > 30 kg/m²,

and type 2 diabetes mellitus (Yadav and Lowenfels 2013). Several CT scoring systems based on either non-enhanced or contrast-enhanced CT studies exist, but according to the current knowledge an early CT is not routinely recommended solely for severity assessment since the predictive accuracy is similar to clinical scoring systems (Bollen et al. 2012).

Clinical scoring systems, such as the Acute Physiology and Chronic Health Evaluation II, the Glasgow-Imrie Score, the Ranson Score, the Harmless Acute Pancreatitis Score, and the Bedside Index for Severity in Acute Pancreatitis, combine several clinical, radiographic, and laboratory findings. However, they are complex and cumbersome to use in clinical practice, and they all offer only moderate accuracy in predicting persistent OD, i.e. SAP, with individual laboratory markers, such as creatinine, providing similar accuracy (Mounzer et al. 2012).

2.6.2 CONVENTIONAL LABORATORY MARKERS

Conventional laboratory markers already in clinical use, such as **creatinine**, **hematocrit** or **calcium**, may predict SAP, but they are considered to reflect rather than predict OD. An increase in serum creatinine or hematocrit level results from intravascular volume depletion. Rising creatinine level that does not respond to adequate fluid resuscitation indicates an increased risk of severe AP (Yang et al. 2014), and after 48 hours of admission, creatinine level $\geq 159 \mu\text{mol/L}$ has been demonstrated to be associated with the development of pancreatic necrosis (Muddana et al. 2009). Hematocrit $\geq 44\%$ on admission predicts SAP (Koutroumpakis et al. 2015). Hypocalcemia may result from sequestration of circulating calcium and albumin into the extracellular space due to increased microvascular permeability (Bhattacharya et al. 1985), and hypocalcemia has been shown to predict SAP on admission to hospital (Mentula et al. 2005) and in ERCP patients within 48 hours of the procedure (Kawa et al. 2000).

2.6.3 MARKERS OF INFLAMMATION

CRP is a major acute-phase protein produced in the liver in response to proinflammatory cytokines such as IL-1 β and TNF. The levels of CRP rise during both bacterial infection and sterile inflammation, but the generic function of CRP in different diseases is not yet fully understood. During inflammation CRP has been shown to opsonize damaged cells, including nuclear breakdown products, such as chromatin and histones, to facilitate their clearance (Abrams et al. 2013). CRP predicts SAP (Puolakkainen et al. 1987), but it peaks with a delay of 48-72 hours after symptom onset, and therefore, it is not usually applicable as a predictor of SAP on admission to

hospital. CRP value ≥ 150 mg/L at 48 hours after onset of symptoms indicates SAP (Dervenis et al. 1999).

Several cytokines and their receptors have been evaluated extensively in AP patients as predictors of SAP. Of these, TNF- α , IL-6, IL-8, and IL-10 are among the most studied markers. **TNF- α** is a crucial cytokine in the pathogenesis of inflammatory reactions, serving as a proximal mediator of inflammation and inducing production of further cytokines, such as IL-6 and IL-8, and it has been shown to predict SAP (Pooran et al. 2003, Surbatovic and Radakovic 2013). However, detecting elevated TNF- α levels in the circulation is challenging due to the rapid hepatic clearance, and also contradictory studies showing no predictive value exist (Paajanen et al. 1995, Digalakis et al. 2009). Many studies report **IL-6** as a predictor of SAP (Viedma et al. 1992, Brivet et al. 1999, Mentula et al. 2005, Fisic et al. 2013), and as an inducer of acute-phase response IL-6 peaks earlier than CRP (Heath et al. 1993). While several studies show **IL-8** to predict SAP (Gross et al. 1992, Shokuhi et al. 2002, Fisic et al. 2013), conflicting results also exist (Mayer et al. 2000). According to a meta-analysis by Aoun et al. (2009), IL-6 and IL-8 seem to perform at an acceptable level, although IL-6 may be better than IL-8 on day 1. **IL-10** is a potent anti-inflammatory cytokine, and several studies show its predictive value in SAP (Brivet et al. 1999, Simovic et al. 1999, Mentula et al. 2005).

In addition to the above-mentioned cytokines, at least **HGF** (Ueda et al. 1996, Espinosa et al. 2011, Sporek et al. 2013), **G-CSF** (Müller et al. 2000), **IL-1 β** , (Mentula et al. 2005), **IL-1 receptor antagonist** (Mentula et al. 2005, Mayer et al. 2000), **growth-related oncogene alpha** (Shokuhi et al. 2002), **IL-18** (Rau et al. 2001), **monocyte chemoattractant protein (MCP) 1** (Rau et al. 2003), **macrophage migration inhibitory factor** (Sakai et al. 2003), **IL-12** (Gregoric et al. 2014), and **IL-15** (Ueda et al. 2007) in humans, and **granulocyte macrophage colony-stimulating factor** in experimental AP (Frossard et al. 2002) have revealed predictive value in SAP. Some studies show also soluble **IL-2 receptor** as a predictor of SAP (Pezzilli et al. 1994, Mayer et al. 2000), but others do not (Kylänpää-Bäck et al. 2001b).

Procalcitonin is already available in clinical practice as a diagnostic marker of sepsis in critically ill patients (Wacker et al. 2013). In patients with AP procalcitonin may predict SAP (Kylänpää-Bäck et al. 2001b, Modifi et al. 2009), but its role is more established in predicting infected pancreatic necrosis and fatal outcome (Rau et al. 2007, Modifi et al. 2009). Serial daily procalcitonin measurements show the procalcitonin levels to fall with clinical improvement both in sepsis (Karlsson et al. 2010) and in AP (Rau et al. 2007, Modifi et al. 2009). However, procalcitonin assay is currently quite expensive, which may hinder its clinical use.

Soluble urokinase-type plasminogen activator receptor, a marker of systemic inflammation, has shown promising results in predicting outcome of critically ill patients (Donadello et al. 2014), being superior to CRP

and procalcitonin (Backes et al. 2012, Uusitalo-Seppälä et al. 2012). Only recently, Nikkola et al. (2017) established it as a predictor of non-mild AP, and Lipinski et al. (2017) as a predictor of SAP, MODS, and fatal AP.

Pentraxin 3, a soluble PRR for innate immunity and an acute-phase protein released from the inflammation site, is another potential research tool in the field of critically ill patients (Zhang et al. 2010), showing similar value as procalcitonin in predicting severe sepsis and fatal outcome (Uusitalo-Seppälä et al. 2013). In patients with AP, pentraxin 3 has been demonstrated to be associated with the severity of AP and to reach peak values before CRP (Kusnierz-Cabala et al. 2013). In the recent study by Deng et al. (2017) that evaluated a vast array of cytokines and conventional biomarkers as predictors of SAP, pentraxin 3 was revealed as an independent predictor of SAP, predicting also development of SAP in patients with SIRS on admission.

Aligned with the theory that endothelial activation and injury are crucial events in the pathogenesis of AP, endothelial markers, such as **intercellular adhesion molecule 1, E-selectin and P-selectin**, have been shown to be elevated in AP and to be associated with OD (Kaufmann et al. 1996, Powell et al. 2001, Zhu and Jiang 2012, Dabrowski et al. 2014, Chen et al. 2017), although contrary findings also exist (Kylänpää-Back et al. 2001b).

In addition, **von Willebrand factor** was recently demonstrated to be associated with MODS in AP (Chen et al. 2017). **CD73/ecto-5'-nucleotidase** is an enzyme that generates adenosine, which dampens inflammation and improves vascular barrier function. The activity of the soluble form of CD73 has been reported to be inversely correlated with the severity of AP and to predict SAP, more importantly already before the clinical signs of OD have developed (Maksimow et al. 2014). The regulator of vascular permeability, **angiopoietin 2**, is associated with persistent OD and SAP (Whitcomb et al. 2010).

Additional inflammatory markers predicting SAP include **CD11b**, an activation marker of monocytes and macrophages (Kylänpää-Bäck et al. 2001a), **complement regulator protein CD59** (Lindström et al. 2008), **matrix metalloproteinase 8** (Nukarinen et al. 2016), and **matrix metalloproteinase 9** (Chen et al. 2006).

Cellular signs of immunosuppression may arise early in SAP and have been revealed to have prognostic value. **Decreased HLA-DR expression** on monocytes is associated with AP severity and predicts the development of OD (Kylänpää-Bäck et al. 2001a, Mentula et al. 2003, 2004). Also **a reduced number of circulating lymphocytes**, inversely proportional to the severity of AP, has been shown (Curley et al. 1993, Pezzilli et al. 1995, Pietruczuk et al. 2006, Dambrauskas et al. 2010).

2.6.4 CELL DEATH MARKERS

Of DAMPs as predictors of SAP, **HMGB1** is the most studied marker (Yasuda et al. 2006, Kocsis et al. 2009, Li et al. 2017), and also a receptor for HMGB1 (but also for several other ligands), namely ***soluble form of receptor for advanced glycation end products*** has been shown to have significantly higher levels in SAP patients with persistent OD compared to milder attack (Lindström et al. 2009). Few clinical studies show also ***cell-free DNA*** (Gornik et al. 2011, Kocsis et al. 2009) and ***histones*** (Liu et al. 2017) as predictors of SAP. Circulating ***nucleosomes*** have been shown to predict OD among a cohort of ICU patients including also patients with AP (Chen et al. 2012).

3 PRESENT INVESTIGATION

3.1 AIMS OF THE STUDY

The main goal of the study was to identify predictors of SAP in AP patients without OD on admission to hospital. Specific aims were as follows:

- I. To evaluate the predictive value of 48 circulating cytokines for SAP in AP patients without OD on admission.
- II. To validate circulating IL-8 and HGF as predictors of SAP in an independent cohort of AP patients without OD on admission, and to investigate whether IL-8 and HGF predict persistent OD in AP patients with OD on admission.
- III. To determine whether circulating nucleosomes predict SAP in AP patients without OD on admission.
- IV. To examine whether similar aberrations in leukocyte signaling occur in sepsis and SAP, and whether they are associated with the severity of AP and the presence of OD.

3.2 MATERIALS AND METHODS

3.2.1 PATIENTS AND HEALTHY CONTROLS

All of the studies include prospective series of non-consecutively enrolled patients. In Studies I-III, all patients had AP, and Study IV comprises both AP and sepsis patients. Healthy volunteers recruited from hospital and laboratory personnel served as controls in Studies II and IV. Study II had 32 healthy controls (20 men and 12 women, median age 46 years, range 21-71), and Study IV 28 healthy controls (19 women and 8 men, median age 35 years, range 24-68). The Surgical Ethical Review Board of the Joint Authority for the Hospital District of Helsinki and Uusimaa approved all study protocols, and a written informed consent was obtained from each patient or the next of kin. Healthy controls gave verbal informed consent.

AP patients were admitted to Helsinki University Hospital within 72 hours (Studies I, III, IV) or within 96 hours (Study II) of symptom onset and were recruited within the first few days after hospital admission. Patients with a known history of chronic pancreatitis were excluded. Sepsis patients were recruited within 48 hours of hospital admission. Exclusion criteria were concomitant malignancy, known immunological deficiency, and in-hospital sepsis. Clinical characteristics and outcome of patients in Studies I-IV are shown in Table 3.

3.2.2 CLASSIFICATION AND DEFINITIONS

Acute pancreatitis

AP diagnosis was based on at least two of the following three features: typical epigastric pain with elevated pancreatic specific plasma amylase level of more than three times the upper limit of normal, and/or typical findings in abdominal CT scan or MRI (Tenner et al. 2013). According to routine hospital practice, early imaging was usually performed without intravenous contrast medium to preserve renal function. The severity of AP was assessed according to the outcome of AP using the Revised Atlanta Classification (Banks et al. 2013) as mild, moderately severe, or severe. The presence of OD on admission was assessed according to the MMS (Marshall et al. 1995), as recommended in the Revised Atlanta Classification (Banks et al. 2013); MMS ≥ 2 from one of the evaluated organ systems indicates OD and MMS < 2 no OD (Table 4). Patient classification in Studies I-IV is shown in Table 5.

Table 3. *Patient characteristics in Studies I-IV.*

Characteristic	Study I, <i>n</i> = 163	Study II, <i>n</i> = 176	Study III, <i>n</i> = 74 ^a	Study IV, <i>n</i> = 18	
				AP, <i>n</i> = 18	Sepsis ^b , <i>n</i> = 14
Study period	6/2003 – 1/2008	3/2011 – 8/2014	6/2003 – 1/2008	2/2010 – 5/2012	
Men, <i>n</i> (%)	120 (74)	122 (69)	57 (77)	11 (61)	8 (57)
Age, years ^c	48 (39-57)	51 (43-64)	48 (39-59)	53 (46-75)	62 (50-65)
Time between onset of symptoms and admission, hours ^c	24 (12-48)	24 (12-48)	24 (12-48)	30 (8-72)	NA
Etiology of AP					
Alcohol, <i>n</i> (%)	113 (69.3)	110 (63)	54 (73)	11	NA
Biliary, <i>n</i> (%)	35 (21.5)	37 (21)	14 (19)	5	NA
Other, <i>n</i> (%)	11 (6.7)	29 (17) ^d	3 (4)	0	NA
Unknown, <i>n</i> (%)	4 (2.5)		3 (4)	2	NA
Mortality, <i>n</i> (%)	8 (4.9)	2 (1)	8 (11)	1 (6)	3 (21)

Abbreviations: AP, Acute pancreatitis; NA, Not applicable.

^aCohort of AP patients included in Study I

^bEtiology of sepsis was pneumonia (*n*=7), intra-abdominal infection (*n*=5), or cervical abscess (*n*=1).

^cValues are in median (interquartile range)

^dOther or unknown

Table 4. *Modified Marshall Score (Marshall et al. 1995, Banks et al. 2013)*

Organ system	Score				
	0	1	2	3	4
Respiratory (Pa2/FiO2), mmHg	>400	301-400	201-300	101-200	≤101
Renal^a (serum creatinine, µmol/L)	<134	134-169	170-310	311-439	>439
Cardiovascular (systolic blood pressure, mmHg) ^b	>90	<90, fluid-responsive	<90, not fluid-responsive	<90, pH<7.3	>90, pH<7.2

For non-ventilated patients, the FiO2 can be estimated as follows: Room air, 21%; 2 l/min, 25%; 4 l/min, 30%; 6-8 l/min, 40%; 9-10 l/min, 50%.

A score of 2 or more in any system indicates the presence of organ dysfunction.

^aA score for patients with pre-existing chronic renal failure depends on the extent of further deterioration of baseline renal function. No formal correction exists for a baseline serum creatinine ≤134 µmol/L.

^bOff inotropic support.

Table 5. *Classification of patients with acute pancreatitis in Studies I-IV*

Study	Severity of acute pancreatitis ^a				
	Mild	Moderately severe		Severe	
	No OD ^b (MMS<2), n	No OD ^b (MMS<2), n	With OD ^b (MMS≥2), n	No OD ^b (MMS<2), n	With OD ^b (MMS≥2), n
I	103	25	10	14	11
II	121	23	9	10	13
III	23	21	6	14	10
IV	9	6	0	2	1

Abbreviations: MMS, Modified Marshall Score; OD, Organ dysfunction.

^aOutcome of acute pancreatitis according to the Revised Atlanta Classification (Banks et al. 2013)

^bOn admission according to the MMS (Marshall et al. 1995)

Sepsis

The diagnosis of sepsis was based on the 1992 sepsis definitions (Bone et al. 1992), but the severity of sepsis was classified according to recent guidelines (Singer et al. 2016) as sepsis and septic shock. Sepsis is defined as a life-threatening OD (an increase in the Sequential organ failure assessment score of 2 points or more) caused by a dysregulated host response to infection (Table 6). Septic shock entails a need for a vasopressor to maintain mean arterial pressure of 65 mmHg or more and serum lactate level greater than 2 mmol/L in the absence of hypovolemia.

Table 6. Sequential Organ Failure Assessment Score (Vincent et al. 1996).

Variable	Score				
	0	1	2	3	4
Respiratory <i>PaO₂/FiO₂, mmHg</i>	>400	≤400	≤300	≤200 ^a	≤100 ^a
Coagulation <i>platelets, x10³/μL</i>	>150	≤150	51-100	21-50	≤20
Liver <i>bilirubin, μmol/L</i>	<20	20-32	33-101	102-204	>204
Cardiovascular, hypotension	No hypotension	MAP <70 mmHg	Dop ≤5 or dob (any dose) ^b	Dop >5, epi≤0.1, or norepi≤0.1 ^b	Dop>15, epi>0.1 or norepi 0.1 ^b
Central nervous system, GCS	5	13-14	10-12	6-9	<6
Renal <i>creatinine, μmol/L or urine output, mL/day</i>	<110	110-170	171-299	300-440 (or <500 mL/d)	>440 (or <200 mL/d)

Abbreviations: Dob, Dobutamine; Dop, Dopamine; Epi, epinephrine; FiO₂, Fraction of inspired oxygen; GCS, Glasgow Coma Score; MAP, Mean arterial pressure; Norepi; Norepinephrine; PaO₂, Arterial partial pressure of oxygen

^aValues are with respiratory support.

^bAdrenergic agents administered for at least one hour (doses given are in μg/kg per minute).

3.2.3 SAMPLING AND ANALYTICAL METHODS

The blood samples were taken on admission after recruitment. For Study I, we used serum, for Studies II and III plasma, and for Study IV whole blood. The samples were stored at -70°C — -80°C to await analyses, except for Study IV, where the samples were analyzed within three hours of sampling.

The predictive values of the conventional markers CRP (Studies I-III), creatinine (Studies I-III), and calcium (Study I) were used for comparison because they have established prognostic value in AP (Puolakkainen et al. 1987, Mentula et al. 2005, Muddana et al. 2009). These values were determined according to the hospital's routine practice (Helsinki University Hospital Laboratory, HUSLAB).

Multiplex detection technique (Study I)

In Study I, the serum levels of 48 cytokines were determined by magnetic bead suspension array (20 µl of each sample) using Multiplex detection technology. Bio-Plex Pro Human Cytokine 21- and 27-plex panels (Bio-Rad Laboratories, Hercules, CA, USA) were used according to the manufacturer's instructions, except that the assay reagents were used at half of their recommended concentrations. The 21-plex panel and the 27-plex panel contained the cytokines shown in Table 7. The samples were analyzed using the Bio-Plex 200 System, and the results were calculated using Bio-Plex Manager 6.0 software (Bio-Rad Laboratories). Values of interferon gamma were below the detectable limit in all patients, and thus, it was excluded from data analyses. In addition, the majority of values for leukemia inhibitory factor, eotaxin, granulocyte colony-stimulating factor, IL-1 α , IL-3, IL-12p40, IL-15, and TNF- β were below detectable limits, and they were scored dichotomically as detectable or undetectable.

Table 7. Cytokine levels analyzed in 21-plex and 27-plex panels using Multiplex detection technology in Study I.

21-plex panel		27-plex panel	
β-NGF	Beta nerve growth factor	FGF	Basic fibroblast growth factor
CTACK	Cutaneous T cell attracting chemokine	Eotaxin	Eotaxin
GRO-α	Growth-regulated oncogene alpha	G-CSF	Granulocyte colony-stimulating factor
HGF	Hepatocyte growth factor	GM-CSF	Granulocyte macrophage colony-stimulating factor
IFN-α2	Interferon alpha 2	MIP-1α	Macrophage inflammatory protein 1 alpha
IL-1α	Interleukin 1 alpha	MIP-1β	Macrophage inflammatory protein 1 beta
IL-2Rα	Interleukin 2 receptor α chain	MCP-1	Monocyte chemoattractant protein 1
IL-3	Interleukin 3	IL-1β	Interleukin 1 beta
IL-12p40	Interleukin 12p40	IL-1Ra	Interleukin 1 receptor antagonist
IL-16	Interleukin 16	IL-2	Interleukin 2
IL-18	Interleukin 18	IL-4	Interleukin 4
LIF	Leukemia inhibitory factor	IL-5	Interleukin 5
M-CSF	Macrophage colony-stimulating factor	IL-6	Interleukin 6
MIF	Macrophage migration inhibitory factor	IL-7	Interleukin 7
MCP-3	Monocyte chemoattractant protein 3	IL-8	Interleukin 8
MIG	Monokine induced by interferon gamma	IL-9	Interleukin 9
SCF	Stem cell factor	IL-10	Interleukin 10
SCGF- β	Stem cell growth factor beta	IL-12p70	Interleukin 12p70
SDF-1α	Stromal cell-derived factor 1 alpha	IL-13	Interleukin 13
TNF-β	Tumor necrosis factor-beta	IL-15	Interleukin 15
TRAIL	TNF-related apoptosis-inducing ligand	IL-17A	Interleukin 17A
		IFN-γ	Interferon gamma
		IP-10	IFN-gamma-induced protein 10
		PDGF-BB	Platelet-derived growth factor, two BB chains
		RANTES	Regulated on activation, normal T cell expressed, and secreted
		TNF-α	Tumor necrosis factor alpha
		VEGF	Vascular endothelial growth factor

Enzyme-linked immunosorbent assay (Studies II-IV)

In Studies II and IV, IL-8 and HGF plasma levels were determined with cytokine-specific enzyme-linked immunosorbent assay (ELISA) using reagents from BD Biosciences, Erembodegem, Belgium (IL-8) and R&DSYSTEMS Europe Ltd., Abingdon, UK (HGF). For Study II, the detection limits and inter-assay coefficients of variation were 0.8 pg/ml and 5.0% for IL-8 and 7.8 pg/ml and 5.6% for HGF, respectively. In 17 of the 176 patients in Study II, the HGF levels were below the detectable limit, and these values were extrapolated as 0.002 ng/ml (one-fifth of the lowest HGF value determined). All IL-8 results fell within the standard range of IL-8 ELISA. In Study IV, the detection limits and inter-assay coefficients of variation were 0.8 pg/ml and 1.4% for IL-8 and 7.8 pg/ml and 7.1% for HGF, respectively.

In Study III, nucleosomes were quantified with Cell Death Detection ELISAPLUS (Roche, Basel, Switzerland) according to the manufacturer's instructions. The results are presented as absorbance units (AUs), and negative values are recorded as zero. Since the plasma samples for this study were collected between June 2003 and January 2008, and the nucleosome measurements were performed in the year 2013, we checked that there was no correlation between sample age and nucleosome level in mild, moderately severe, or severe AP (Spearman's $r=0.31$, $p=0.153$; $r=0.02$, $p=0.928$; and $r=0.10$, $p=0.631$, respectively).

Flow cytometry (Study IV)

In Study IV, the target molecule phosphorylation was determined by means of phosphospecific whole blood flow cytometry, the method first described by our group in 2008 concerning STAT1 phosphorylation in circulating monocytes (Vakkila et al. 2008). In the studies by Oiva et al. (2010a, 2010b, 2013), this method was used to investigate NF- κ B, STAT1, STAT3, STAT5, STAT6, and p38 and ERK1/2 MAPKs, in monocytes, lymphocytes, and neutrophils of SAP patients. In the current study, we chose to determine NF- κ B, STAT1, STAT3, and ERK1/2 MAPK phosphorylation in non-stimulated and stimulated monocytes, lymphocytes, and neutrophils of sepsis and AP patients. The stimulation strategy was as follows: NF- κ B stimulation by *E. coli* and LPS in monocytes and neutrophils and by TNF in each of the three cell types; STAT1 and STAT3 by IL-6 in each cell type, and ERK1/2 MAPK by the combination of PMA and Ca^{2+} -ionophore A_{23187} in each cell type.

A 4-ml venous blood sample was collected into a Falcon polypropylene tube (Becton Dickinson, Lincoln Park, NJ, USA) supplemented with pyrogen-free citrate phosphate dextrose (Baxter Health Care Ltd., Norfolk, England, 0.1 ml/ml blood) and kept at room temperature until stimulations within three hours of sampling. Each patient sample was compared with a sample obtained from a healthy volunteer within two weeks.

Monoclonal antibodies against cell surface structures used for surface marker staining comprised fluorescein isothiocyanate conjugated anti-CD14 (clone clone M ϕ P9 IgG_{2b}) and phycoerythrin conjugated anti-HLA-DR (clone

L243, IgG2a) and its isotype control (mouse IgG2a). Monoclonal antibodies for intracellular phosphospecific labeling included AlexaFluor647-conjugated anti-pNF- κ B p65 (pS529) (clone K10-895.12.50, IgG2b), anti-STAT1 (pY701) (clone 4a, IgG2a), anti-ERK1/2 (pT202/pY204) (clone 20A, IgG1), and phycoerythrin conjugated anti-STAT3 (pY705) (clone 4/P-STAT3, IgG2a). All antibodies were from Becton-Dickinson Biosciences (San Jose, CA, USA).

The recombinant cytokines TNF and IL-6 were obtained from R&D (Minneapolis, MN, USA). PMA and Ca^{2+} -ionophore A_{23187} , and *E.coli* O11:B4 LPS were from Sigma (St. Louis, MO, USA). Whole *E. coli* bacteria were purchased from The National Institute for Health and Welfare (Helsinki, Finland), grown in brain heart infusion medium, pelleted, and washed twice. A diluted culture was made to quantify viable bacteria. The bacteria were pelleted, resuspended in glycerol-tryptone soya broth medium, and stored at -70°C . The stimuli were diluted in phosphate-buffered saline before use.

The blood sample was divided into 100- μl aliquots in Falcon polystyrene tubes (Becton Dickinson, Lincoln Park, NJ, USA). Next, the tubes were supplemented either with TNF 10 ng/ml (final concentration), IL-6 100 ng/ml, PMA + A_{23187} each 1 $\mu\text{g}/\text{ml}$ for 5 min, LPS 100 ng/ml, or *E. coli* (50 cells/leukocyte) for 10 min at 37°C . Reference tubes were left without stimulus.

After incubations, leukocyte fixation, red cell lysis, and leukocyte permeabilization were performed according to Becton Dickinson Phosflow Protocol III for Human Whole Blood. After permeabilization, the samples were washed with Beckton Dickinson Pharmingen Stain Buffer and resuspended in 100 μl of the buffer. Aliquots of AlexaFluor647- and phycoerythrin labelled phosphospecific Abs and anti-CD3-PerCP were added. The samples were incubated for 20 min in the dark at room temperature, washed, resuspended in 300 μl of the buffer, stored in the dark on ice, and run on the flow cytometer within three hours.

Data acquisition and analysis were carried out by FACSCanto II flow cytometer with FACSDiva software (Becton Dickinson Sciences, San Jose, CA, USA). The anti-CD14- fluorescein isothiocyanate label was used for identifying monocytes. Neutrophils and lymphocytes were first delineated according to their light scattering properties, and strongly CD14-fluorescein isothiocyanate positive events were excluded using electronic gates. AlexaFluor647 and phycoerythrin histograms were developed from stimulated and non-stimulated monocytes, neutrophils, and lymphocytes.

Flow cytometric data on the intracellular targets were determined as follows: First, the median of relative fluorescence units (RFU) of all cells of a given leukocyte population indicates whether the target molecule phosphorylation is aberrant relative to healthy controls. The aberration can be caused by a different size of the normally responding cellular subpopulation, which is shown by the proportion (%) of positively fluorescing cells, or a different capability of the responding cells to raise the target phosphorylation, which is shown by the average RFU of the proportion of positive fluorescing

cells. The proportion of the positively fluorescing cells and their median RFU were measured using a threshold method. In brief, an electronic gate covering the brightest fluorescing cells was set manually to comprise less than but as close as possible to 5% of the cells in the non-stimulated sample, and the same gate was used to determine the proportion of positively fluorescing cells in the respective stimulus-treated sample. Thus, values < 5% indicate that the cells have not responded to the stimulus.

Constitutive STAT3 phosphorylation was measured as the proportion of constitutively STAT3-phosphorylated cells (pSTAT3+ %) and their median RFU. In brief, a marker was set on each cell population histogram of the healthy subject's sample so that it includes less than but as close as possible to 5% of the brightest events, and the markers were then copied to the respective patient's sample histograms.

The proportion of HLA-DR-positive monocytes was determined as described previously (Kylänpää-Bäck et al. 2001a).

3.2.4 STATISTICAL ANALYSIS

Statistical analyses were performed using IBM SPSS® Statistic versions 19 and 22 (SPSS, Chicago, IL, USA) statistical software. Because of the skewness of the data, non-parametric tests were used. The results are given as median and interquartile range (IQR) or range if $n \leq 3$, or number of patients and percentages. Correlations between two continuous variables were tested using Spearman rank correlation (r = correlation coefficient). Comparisons for continuous variables between two groups were made using Mann-Whitney U test. Jonckheere-Terpstra test for trend test was used for comparisons between three ordered groups. P values ≤ 0.05 were considered significant, and double-sided tests were used. P values of multiple comparisons were corrected using the Bonferroni method.

The predictive value of a biomarker (Studies I-III) and a combined regression model (Studies I and II) was first assessed using a receiver operator characteristic (ROC) curve and calculating the area under the curve (AUC), ranging from 0-1. AUC 1.0 is a perfect test, and AUC 0.5 is similar to tossing a coin. Then, the optimal cut-off value of a biomarker was determined from the curve. An optimal predictive marker has both high specificity and high sensitivity. Since ICU beds are limited in real hospital life and should be allocated wisely, we figured that a useful marker should have high specificity, preferably $\geq 90\%$. We therefore chose a "clinically optimal" cut-off value from the ROC curve where the longest increase in the sensitivity of the slope declines, and the specificity is $\geq 90\%$. For the optimal cut-off value, we calculated, apart from specificity and sensitivity, positive and negative likelihood ratio and diagnostic odds ratio (Glas et al. 2003) with 95% confidence intervals (Newcombe 1998). Univariate logistic regression analysis was used for identifying significant predictors of SAP, and multivariate logistic

regression analysis with forward conditional stepping was used, when appropriate, to identify independent predictors of SAP. Here, $p < 0.005$ inclusion and $p > 0.10$ removal criteria were used to select a variable into the model.

3.3 RESULTS

3.3.1 PREDICTORS OF DEVELOPMENT OF SEVERE ACUTE PANCREATITIS (I-III)

3.3.1.1 *Circulating cytokines (I)*

Of the 48 cytokines (Table 7) screened in the whole AP cohort as potential predictors of SAP on admission, 14 showed significantly higher levels in SAP than in non-SAP when Bonferroni-adjusted p values were used ($p < 0.001$) (Table 8). A significant difference ($p < 0.05$) between the non-SAP and SAP group was found also for CRP, creatinine, and calcium (data not shown). The levels of all 14 cytokines correlated with each other (Spearman's $r \geq 0.21$ for all, $p \leq 0.007$), and the correlation was especially strong between IL-6 and either IL-8, G-CSF, MCP-1, HGF, or macrophage colony-stimulating factor ($r > 0.6$, $p < 0.001$). The median time between onset of symptoms and hospital admission was 24 hours in all AP patients (Table 3), and it did not differ between non-SAP and SAP groups (data not shown, $p = 0.450$).

A forward stepping logistic regression analysis of those 14 cytokines revealed only IL-6 and HGF as independent predictors of SAP ($p = 0.006$ and $p < 0.001$, respectively). When calcium, creatinine, and CRP were added into the logistic regression analysis with IL-6 and HGF, calcium was also an independent predictor of SAP ($p = 0.023$). The AUCs of IL-6, HGF, and calcium to predict SAP were 0.87 (95% CI 0.81-0.94), 0.81 (0.72-0.90), and 0.81 (0.70 to 0.91), respectively. Using the clinically optimal cut-off value (specificity $\geq 90\%$), IL-6 > 501.6 pg/mL, HGF > 3020.1 pg/mL, and calcium < 1.91 mmol/L predicted SAP with sensitivities of 48% (30-67%), 60% (41-77%), and 58% (36-77%), positive likelihood ratios of 7.4 (3.5-15.6), 8.3 (4.2-16.3), and 8.5 (4.1-17.8), and diagnostic odds ratios of 13.2 (4.7-37.3), 19.2 (6.9-53.6), and 18.8 (6.0-58.4), respectively.

The patients presenting without OD (MMS < 2) were analyzed further. Of these, 14/142 developed SAP during hospitalization. The median time between onset of symptoms and hospital admission was 24 hours (IQR 12- 48 hours), which did not differ significantly between non-SAP and SAP groups (data not shown, $p = 0.161$). Of the 14 cytokines showing a significant difference between non-SAP and SAP patients in the whole AP cohort, only HGF, IL-8, and G-CSF

Table 8. Levels of 14/48 cytokines showing significant difference between non-SAP and SAP among all patients and the respective cytokine levels among patients without organ dysfunction on admission.

Cytokine, pg/mL	All patients, n=163			No OD on admission, n=142		
	Non-SAP, n=138	SAP, n=25	p value ^a	Non-SAP, n=128	SAP, n=14	p value ^a
G-CSF	119.7 (66.6-198.2)	260.2 (132.5-1011.8)	<0.001	118.4 (66.6-174.3)	234.0 (151.2-531.1)	0.0007
HGF	1055.5 (764.7-1730.6)	3613.0 (2055.3-6348.8)	<0.001	986.6 (753.6-1501.6)	2202.6 (1554.3-3305.5)	<0.001
IL-8	26.6 (19.3-41.9)	82.4 (46.4-115.6)	<0.001	25.4 (18.0-37.6)	59.7 (32.0-102.8)	<0.001
GRO α	58.8 (35.7-92.3)	128.2 (98.4-169.1)	<0.001	54.5 (33.2-82.5)	118.5 (62.4-158.4)	0.002 (NS)
IL-2R α	214.2 (150.6-316.7)	483.3 (385.6-673.3)	<0.001	209.5 (145.5-290.4)	449.7 (228.1-504.3)	0.0015 (NS)
IL-6	59.7 (15.3-202.1)	428.9 (138.5-1796.3)	<0.001	49.4 (14.7-185.6)	234.5 (66.2-1931.3)	0.0013 (NS)
IL-18	139.6 (91.6-187.1)	202.8 (151.8-305.4)	<0.001	131.5 (90.7-181.5)	162.4 (135.3-201.6)	0.062 (NS)
LIF ^b	0 (0)	1 (0-1)	<0.001	0 (0)	1 (0-1)	0.002 (NS)
M-CSF	14.5 (8.1-28.1)	40.2 (18.6-68.6)	<0.001	12.4 (5.5-21.5)	28.7 (12.2-52.0)	0.007 (NS)
MCP-1	49.0 (21.0-111.2)	125.0 (64.6-199.6)	<0.001	47.6 (30.0-100.6)	92.1 (36.3-185.2)	0.032 (NS)
MCP-3	17.4 (0.6-53.6)	54.6 (24.3-98.7)	<0.001	16.3 (0.2-47.9)	52.0 (16.5-102.1)	0.006 (NS)
b-NGF	5.8 (3.7-9.1)	11.3 (7.4-15.9)	<0.001	5.5 (3.4-8.5)	10.4 (4.5-15.5)	0.007 (NS)
SCF	114.1 (85.2-147.0)	155.8 (109.2-256.0)	<0.001	111.6 (82.0-143.3)	148.5 (107.3-218.9)	0.012 (NS)
SDF-1 α	87.6 (53.5-151.5)	163.6 (104.4-226.3)	<0.001	86.8 (52.8-148.5)	171.3 (85.1-234.8)	0.010 (NS)

Abbreviations: NS, Not significant; MMS, Modified Marshall Score; OD, Organ dysfunction; SAP, Severe acute pancreatitis. For the list of the cytokine abbreviations, see Table 7. ^ap<0.001 is significant after Bonferroni correction. ^b0=undetectable value, 1=detectable value. Data denotes median (interquartile range).

showed a significant difference in patients without OD on admission ($MMS < 2$) (Table 8). Of the conventional markers (CRP, creatinine, calcium), only calcium levels differed significantly ($p < 0.001$) between non-SAP and SAP patients (data not shown). In the ROC curve analysis HGF, IL-8, and G-CSF predicted SAP with AUCs of 0.83, 0.79, and 0.78, respectively (Figure 6, Table 9). Calcium performed equally well, with AUC of 0.74 (Table 9). Here, we used the cut-off values obtained from the whole patient cohort to determine the statistical performances of a biomarker (Table 9). Due to the limited number of SAP patients ($n=14$) in this subgroup, we did not perform a multivariate analysis, but instead determined how many of the SAP patients could be identified using a single cytokine or a combination of them. HGF and IL-8 performed equally, both correctly identifying 4/14 SAP patients. G-CSF identified one additional SAP patient (5/14). The best combination of the markers was that of IL-8 and HGF, meaning that if either of the markers was above the cut-off level, 8/14 of the SAP patients were correctly identified as having SAP before they developed OD.

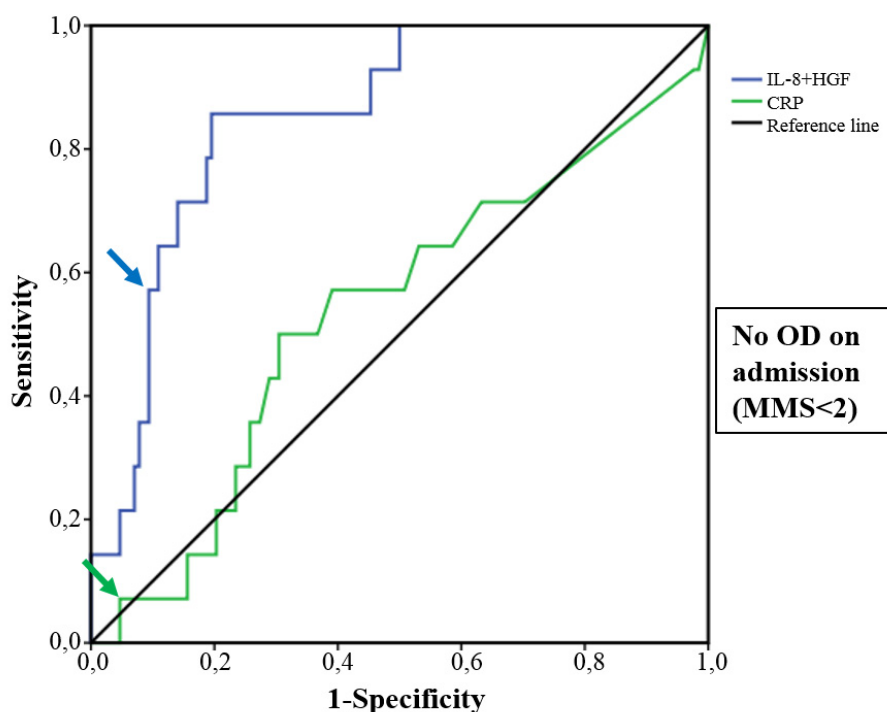


Figure 6 Receiver operating characteristic curves for the combined logistic regression model of IL-8 and HGF, and CRP in predicting severe acute pancreatitis among patients without OD on admission ($n=142$). The arrows show the clinically optimal cut-off values (specificity $\geq 90\%$) used to calculate the statistical performance of the biomarkers for Table 9. *Abbreviations:* CRP, C-reactive protein; HGF, Hepatocyte growth factor; IL, Interleukin; MMS, Modified Marshall Score; OD, Organ dysfunction.

3.3.1.2 IL-8 and HGF (II)

In Study II, the predictive value of IL-8 and HGF for development of SAP was evaluated in another cohort of AP patients and among healthy controls using a cytokine-specific ELISA. The median levels of circulating IL-8 and HGF were very low among healthy controls (7.0 pg/mL, IQR 6.1-8.9 and 0.002 ng/mL, IQR 0.002-0.18, respectively), and they differed significantly ($p < 0.001$) from the patients with AP (32.1 pg/mL, IQR 18.5-71.2 and 0.56 ng/mL, IQR 0.14-0.81, respectively). IL-8 and HGF levels were associated with the severity of AP ($p < 0.001$ for both) and correlated with each other (Spearman's $r = 0.514$, $p < 0.001$).

Of the 176 patients, 154 had no OD (MMS < 2) on admission, but of these, 10 developed SAP during hospitalization. The median time between onset of symptoms and hospital admission was 24 hours (IQR 12-48 hours) and it did not differ significantly between non-SAP and SAP groups (data not shown, $p = 0.835$). A significant difference ($p < 0.05$) between non-SAP and SAP was found for IL-8 and HGF (data not shown). In the ROC curve analysis, IL-8, HGF, and their combined logistic regression model predicted SAP with AUCs of 0.73, 0.79, and 0.82, respectively (Figure 8, Table 9).

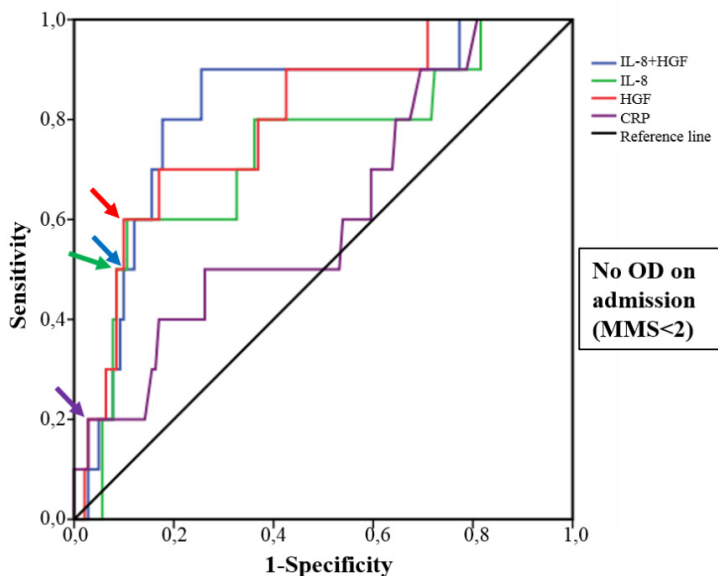


Figure 7 Receiver operating characteristic curves for the combined logistic regression model of IL-8 and HGF, IL-8, HGF, and CRP in predicting severe acute pancreatitis among patients without OD on admission ($n=154$). The arrows show the clinically optimal cut-off values (specificity $\geq 90\%$) used to calculate the statistical performance of the biomarkers for Table 9. *Abbreviations:* CRP, C-reactive protein; HGF, Hepatocyte growth factor, IL, Interleukin, MMS, Modified Marshall Score; OD, Organ dysfunction.

The diagnostic performances of the markers at the clinically optimal cut-off level are presented in Table 9. The results show that combining the markers did not improve the model. The univariate regression analysis revealed IL-8 > 120.9 pg/mL, HGF > 1.66 ng/mL, and CRP > 268 mg/L, but not creatinine (cut-off > 100 μ mol/L), as significant predictors of SAP, with the odds ratios of 11.0 (95% CI 2.8-43.4), 13.6 (3.4-54.1), 8.8 (1.4-55.1), and 3.8 (0.7-20.3), respectively.

3.3.1.3 Circulating nucleosomes (III)

In the whole AP cohort, the median time between onset of symptoms and hospital admission was 24 hours (Table 3), and it did not differ significantly between non-SAP or SAP patients (data not shown, $p=0.833$). Nucleosome levels increased along with the severity of AP ($p<0.001$) and were significantly higher ($p=0.002$) in SAP (0.30 AU, IQR 0.17-0.45) than in non-SAP (0.07 AU, IQR 0.02-0.22). Nucleosome levels were also significantly higher ($p=0.019$) in non-survivors (0.38 AU, IQR 0.19-0.77) than in survivors (0.09 AU, IQR 0.02-0.27).

Nucleosomes, CRP, and creatinine predicted SAP with AUC of 0.72 (95% CI 0.58-0.86), 0.77 (0.65-0.87), and 0.67 (0.58-81), respectively. Using the clinically optimal cut-off value (specificity $\geq 90\%$), nucleosomes > 0.57 AU, creatinine > 110 μ mol/L, and CRP > 264 mg/L predicted SAP with sensitivities of 21% (9-40%), 46% (28-65%), and 33% (18-53%), positive likelihood ratios of 5.2 (1.1-24.9), 5.2 (2.0-13.4), and 3.8 (1.4-10.4), and diagnostic odds ratios of 6.3 (1.1-35.4), 8.8 (2.6-29.8), and 5.2 (1.5-18.1), respectively. In the forward stepping multivariate logistic regression analysis (gender-adjusted), only creatinine ≥ 110 μ mol/L was an independent predictor of SAP with an odds ratio of 6.2 (95% CI 1.7-21.9) ($p=0.005$).

Among the 58 patients without OD (MMS<2) on admission, 14 developed SAP during hospitalization. The median time between onset of symptoms and hospital admission was 24 hours (IQR 12- 48 hours), and it did not differ significantly between non-SAP and SAP patients (data not shown, $p=0.101$). Nucleosomes, creatinine and CRP predicted SAP with AUCs of 0.65, 0.67, and 0.54, respectively (Figure 8, Table 9). The diagnostic performances of the nucleosomes, creatinine, and CRP are shown in Table 9. The forward stepping multivariate logistic regression analysis (gender-adjusted) of the biomarkers revealed only nucleosomes as an independent predictor of SAP with an odds ratio of 1.2 (95% CI 1.2-899.5) ($p=0.038$).

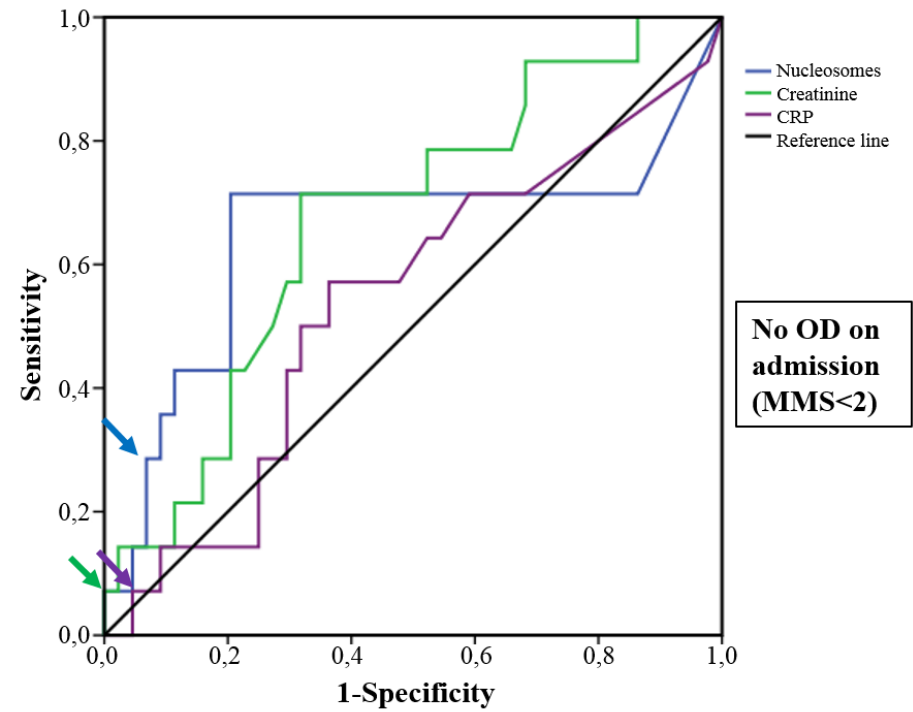


Figure 8 Receiver operating characteristic curves for nucleosomes, creatinine, and CRP in predicting severe acute pancreatitis among patients without OD on admission ($n=58$). The arrows show the clinically optimal cut-off values (specificity $\geq 90\%$) used to calculate the statistical performance of the biomarkers for Table 9. Abbreviations: CRP, C-reactive protein; MMS, Modified Marshall Score; OD, Organ dysfunction.

Table 9. Statistical performance of biomarkers among patients without organ dysfunction on admission.

Predictor	Cut-off	AUC	Sensitivity, %	Specificity, %	LR+	LR-	DOR
Study I, n=142; SAP, n=14							
HGF	3020.1 pg/mL	0.83 (0.73-0.92)	29 (12-55)	95 (90-98)	6.1 (2.0-19.0)	0.8 (0.5-1.1)	8.1 (2.0-33.6)
IL-8	88.1 pg/mL	0.79 (0.68-0.90)	26 (12-55)	95 (90-98)	6.1 (2.0-19.0)	0.8 (0.5-1.1)	8.1 (2.0-33.6)
G-CSF	477.7 pg/mL	0.78 (0.65-0.90)	36 (16-61)	96 (91-98)	9.1 (3.0-27.7)	0.7 (0.5-1.0)	13.7 (3.3-56.1)
IL-8+HGF	88.1 pg/mL or 3.0 ng/mL	0.85 (0.77-0.94)	57 (33-79)	92 (86-96)	7.3 (3.5-15.5)	0.5 (0.3-0.9)	15.7 (4.6-54.4)
Calcium ^a	1.91 mmol/L	0.74 (0.60-0.88)	36 (15-65)	94 (89-97)	6.4 (2.2-18.5)	0.7 (0.4-1.1)	9.5 (2.2-40.2)
Creatinine	141 µmol/L	0.62 (0.45-0.79)	7 (1-32)	99 (96-100)	9.1 (0.6-37.2)	0.94 (0.91-1.08)	9.7 (0.6-164.2)
CRP	227 mg/L	0.54 (0.37-0.70)	7 (1-32)	95 (90-98)	1.5 (0.2-11.8)	0.97 (0.2-11.8)	1.6 (0.2-14.0)
Study II, n=154; SAP, n=10							
HGF	1.66 ng/mL	0.79 (0.66-0.93)	60 (26-88)	90 (84-94)	6.0 (3.0-12.3)	0.4 (0.2-1.0)	13.6 (3.4-54.1)
IL-8	120.9 pg/mL	0.73 (0.56-0.91)	50 (19-81)	92 (86-96)	6.0 (2.6-13.7)	0.6 (0.3-1.0)	11.0 (2.8-43.4)
IL-8+HGF	NA	0.82 (0.68-0.95)	50 (24-76)	90 (84-94)	5.0 (2.3-11.1)	0.6 (0.3-1.0)	9.1 (2.3-35.2)
CRP	268 mg/L	0.62 (0.44-0.80)	20 (6-51)	97 (93-99)	7.2 (1.5-34.7)	0.8 (0.6-1.1)	8.8 (1.4-55.1)
Creatinine	100 µmol/L	0.59 (0.40-0.78)	20 (6-51)	94 (89-97)	3.2 (0.8-12.9)	0.9 (0.6-1.2)	3.8 (0.7-20.3)
Study III, n=58; SAP, n=14							
Nucleosomes	0.39 AU	0.65 (0.44-0.85)	29 (12-55)	93 (82-98)	4.2 (1.1-16.5)	0.8 (0.6-1.1)	5.5 (1.1-28.4)
Creatinine	139 µmol/L	0.67 (0.51-0.83)	14 (4-40)	98 (90-100)	7.3 (0.7-74.6)	0.9 (0.7-1.09)	8.3 (0.7-99.7)
CRP	227 mg/L	0.54 (0.36-0.72)	14 (4-40)	92 (82-97)	1.8 (0.4-8.9)	0.9 (0.7-1.2)	2.0 (0.3-12.0)

Abbreviations: AUC, Area under the curve; CRP, C-reactive protein; DOR, Diagnostic odds ratio; HGF, Hepatocyte growth factor; G-CSF, Granulocyte colony-stimulating factor; IL, Interleukin; LR, Likelihood ratio, NA, Not assessed, SAP, Severe acute pancreatitis. ^an=134; SAP, n=11. 95% confidence intervals are presented in parenthesis.

3.3.2 PREDICTORS OF PERSISTENT ORGAN DYSFUNCTION IN ACUTE PANCREATITIS (I-III)

In Study I, 21/163 patients had OD ($\text{MMS} \geq 2$) on admission. The median time between onset of symptoms and hospital admission was 33 hours (IQR 24-54 hours) in patients with transient OD and 48 hours (24-72 hours) in those with persistent OD, but the difference was not significant ($p=0.349$). Of the 14 cytokines showing significant difference between non-SAP and SAP in the whole AP patient cohort (Table 8), only the HGF levels differed significantly between patients with persistent OD ($n=11$) and transient OD ($n=10$), $p=0.007$; the difference was not significant after applying the Bonferroni method ($p \leq 0.001$ is significant) (Table 10).

Table 10. Biomarker levels among patients with organ dysfunction ($\text{MMS} \geq 2$) on admission.

Biomarker	Transient OD (<48 hours)	<i>n</i>	Persistent OD (≤ 48 hours)	<i>n</i>	<i>p</i> value
Study I					
HGF	2212.2 (1855.4-4703.2)	10	5293.5 (3933.2-8194.2)	11	0.006 ^a (NS)
IL-8	69.5 (41.4-124.2)	10	102.3 (82.4-217.7)	11	0.061 ^a (NS)
Study II					
HGF	0.58 (0.38-2.3)	9	1.56 (1.06-5.33)	13	0.030
	52.7 (20.6-95.2)	9	165.6 (102.6-741.7)	13	0.004
Study III					
Nucleosomes	0.41 (0.18-0.71)	6	0.39 (0.24-0.75)	10	0.635 (NS)

Abbreviations: HGF, Hepatocyte growth factor; IL, Interleukin; NS, Not significant; OD, Organ dysfunction.

^aIn study I $p \leq 0.001$ is significant when Bonferroni correction is applied.

Data denote median (interquartile range).

In Study II, 22/176 patients had OD ($\text{MMS} \geq 2$) on admission. Both IL-8 and HGF levels were significantly higher in patients with persistent OD ($n=13$) relative to those with transient OD ($n=9$) (Table 10). In the ROC curve analysis, IL-8, HGF, and their combined logistic regression model predicted SAP with AUCs of 0.86, 0.78, and 0.84, respectively (Figure 9, Table 11), but CRP and creatinine did not, with respective AUCs of only 0.43 and 0.36. Combining IL-8 and HGF did not improve the model (Figure 9, Table 11). At the clinically optimal cut-off level, IL-8 showed a better sensitivity than HGF, and in the univariate analysis only IL-8 (cut-off > 130.9 pg/mL) was an independent predictor of SAP with an age-adjusted odds ratio of 40.3 (95% CI 1.7-938.2). In patients with persistent OD, the median time between onset of symptoms

and hospital admission was significantly shorter than in those with transient OD (12 hours; IQR 9-12 hours vs. 24 hours; IQR 18-59; $p=0.036$). However, in the univariate analysis, it was not a statistically significant predictor of SAP (OR 0.948; 95% CI 0.898-1.001; $p=0.056$).

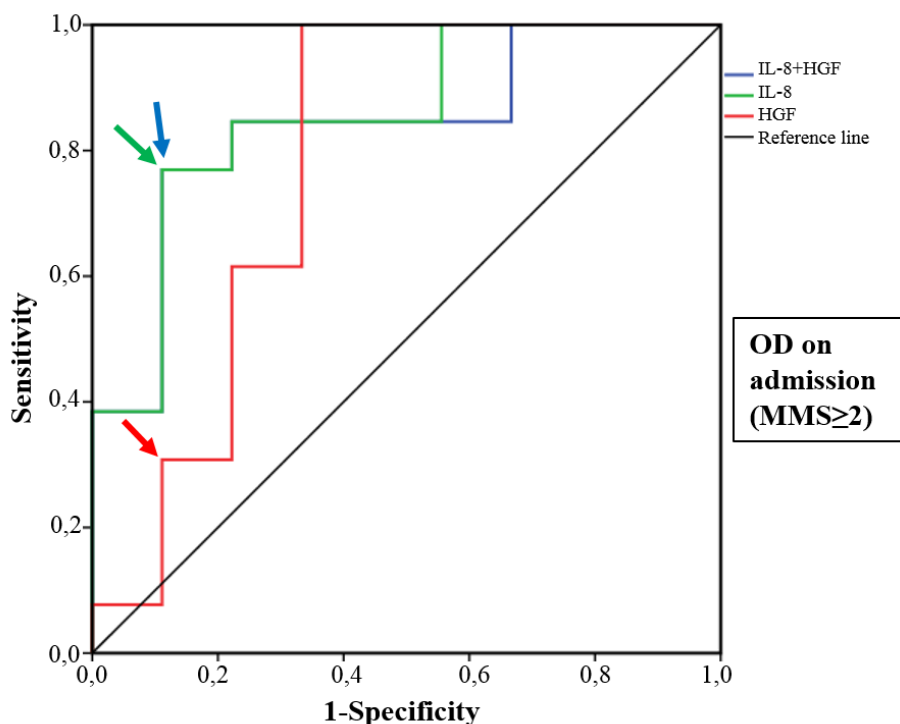


Figure 9 ROC curve for IL-8, HGF, and their combination to predict severe acute pancreatitis among patients with OD on admission ($n=22$) in Study II. The arrows show the clinically optimal cut-off values (specificity $\geq 90\%$) used to calculate the statistical performance of the biomarkers for Table 11. *Abbreviations:* HGF, Hepatocyte growth factor; IL, Interleukin; MMS, Modified Marshall Score; OD, Organ dysfunction.

In Study III, 16/74 patients presented with OD ($MMS \geq 2$). The median time from onset of symptoms to hospital admission did not differ significantly between patients with transient OD ($n=6$) and patients with persistent OD ($n=10$) (data not shown, $p=0.792$). There was also no significant difference in the nucleosome levels (Table 10).

Table 11. Statistical performance of IL-8 and HGF to predict persistent organ dysfunction among patients presenting with organ dysfunction in Study II.

Cytokine	Cut-off	AUC	Sensitivity, %	Specificity, %	+LR	-LR	DOR
IL-8	130.9 pg/mL	0.88 (0.69-1.0)	77 (50-92)	89 (57-98)	6.9 (1.1-45.0)	0.3 (0.09-0.72)	26.7 (2.3-308.0)
HGF	4.07 ng/mL	0.78 (0.55-1.0)	31 (13-58)	89 (57-98)	2.8 (0.4-20.9)	0.8 (0.5-1.2)	3.6 (0.3-38.8)
IL-8+HGF	NA	0.84 (0.66-1.0)	77 (50-92)	89 (57-98)	6.9 (1.1-45.0)	0.3 (0.1-0.7)	26.7 (2.3-308)

Abbreviations: HGF, Hepatocyte growth factor; IL, Interleukin; AUC, Area under the curve; DOR, Diagnostic odds ratio; LR, Likelihood ratio; NA, Not assessed
95% confidence intervals are presented in parentheses.

3.3.3 LEUKOCYTE SIGNALING PROFILES IN SEPSIS AND ACUTE PANCREATITIS (IV)

In AP patients, the median time between onset of symptoms and hospital admission was 30 hours (Table 3), and it did not differ significantly between mild, moderately severe AP, or SAP (data not shown, $p=0.309$). Although on hospital admission only one SAP patient had OD (Table 5), at blood sampling (which occurred 22-44 hours later) all three of them had OD ($MMS \geq 2$). Creatinine, IL-8, and HGF levels increased along with the severity of AP ($p < 0.05$ for all), unlike CRP ($p=0.296$) (data not shown).

In sepsis patients, the information regarding the time between onset of symptoms and hospital admission was not routinely reported in the medical charts. These patients were admitted to the ICU (where sampling occurred) within 32 hours of hospital admission (median 7 hours).

NF- κ B

In monocytes and lymphocytes of both sepsis and AP patients, NF- κ B signaling activity was depressed after ex vivo stimulation, shown both as a lower proportion of positively fluorescing cells (pNF- κ B+%) and their lower pNF- κ B fluorescence intensities compared to healthy controls (Table 12). In AP patients' neutrophils, pNF κ B+% was lower than in healthy controls.

STAT1

In IL-6 stimulated monocytes of both sepsis and AP patients, pSTAT1+% and the respective pSTAT1 fluorescence intensity were lower than in healthy controls (Table 13). In IL-6 stimulated lymphocytes, pSTAT1+% was lower in sepsis and AP patients than in healthy controls.

STAT3

The STAT3 signaling pathway was constitutively activated without stimulus in monocytes, neutrophils, and lymphocytes of both sepsis and AP patients, but not in healthy controls (Table 13). In IL-6 stimulated samples of sepsis and AP patients, the pSTAT3+% and the corresponding fluorescence intensity were lower in monocytes and higher in neutrophils, and in IL-6 stimulated lymphocytes of sepsis patients, pSTAT3+% was higher than in controls.

ERK1/2 MAPK

In PMA+A₂₃₁₈₇ stimulated monocytes of both sepsis and AP patients, the pERK1/2 fluorescing intensity was lower than in healthy controls (Table 14). In PMA+A₂₃₁₈₇ stimulated neutrophils of sepsis patients, pERK1/2+% was higher than in healthy controls. In the lymphocytes of AP patients, pERK1/2+% and the corresponding fluorescence intensity were lower than in healthy controls.

Table 12. *NF-κB signaling after ex vivo stimulation in monocytes, neutrophils, and lymphocytes of healthy controls and sepsis and acute pancreatitis patients.*

Leukocyte population/ Signal (stimulus), proportion (%) or RFU of positively fluorescing cells	Healthy controls, n=28	Sepsis, n=14	p value ^a , MWU	Acute pancreatitis, n=18	p value ^b , MWU
Monocytes					
NF-κB (TNF), %	81.7 (69.3-84.2)	26.5 (18.4-48.4)	<0.001	23.7 (18.3-41.5)	<0.001
NF-κB (TNF), RFU	1505 (1290-1736)	749 (676-907)	<0.001	846 (729-977)	<0.001
NF-κB (LPS), %	66.5 (50.9-81.9)	22.8 (11.0-36.4)	<0.001	23.5 (13.2-33.9)	<0.001
NF-κB (LPS), RFU	1250 (1049-1487)	731 (624-903)	<0.001	781 (722-1055)	<0.001
NF-κB (<i>E. Coli</i>), %	87.0 (77.8-91.5)	30.2 (13.3-60.9)	<0.001	43.2 (28.3-58.1)	<0.001
NF-κB (<i>E. Coli</i>), RFU	1637 (1495-2031)	747 (627-898)	<0.001	867 (761-1144)	<0.001
Neutrophils					
NF-κB (TNF), %	13.0 (9.3-19.9)	12.3 (9.1-17.6)	0.501	9.5 (6.9-13.0)	0.022
NF-κB (TNF), RFU	949 (780-1089)	866 (726-1028)	0.228	945 (766-1136)	0.910
Lymphocytes					
NF-κB (TNF), %	43.2 (33.7-52.3)	15.3 (8.0-20.6)	<0.001	9.5 (7.6-20.4)	<0.001
NF-κB (TNF), RFU	647 (582-718) ^c	440 (387-557)	<0.001	462 (396-563)	<0.001

Abbreviations: *E. coli*, *Escherichia coli*; LPS, Lipopolysaccharide; MWU, Mann-Whitney U; RFU, Relative fluorescence unit; TNF, Tumor necrosis factor.

^abetween healthy controls and sepsis, ^bbetween healthy controls and acute pancreatitis, ^cn = 26. Data denote median (quartiles).

Table 13. STAT1 and STAT3 signaling after ex vivo stimulation and constitutive STAT3 phosphorylation without stimulus in monocytes, neutrophils, and lymphocytes of healthy controls and sepsis and acute pancreatitis patients.

Leukocyte population/Signal (stimulus), proportion (%) or RFU of positively fluorescing cells	Healthy controls, n=28	Sepsis, n=14	p value ^a ,		Acute pancreatitis, n=18		p value ^b ,	
				MWU		MWU		MWU
Monocytes								
STAT1 (IL-6), %	52.2 (39.3-60.0)	4.9 (4.0-7.3)	<0.001		4.8 (4.1-10.6)		<0.001	
STAT1 (IL-6), RFU	751 (703-873)	665 (592-795)	0.030		670 (615-801)		0.030	
STAT3 constitutive (no stimulus), %	< 5 %	67.2 (52.9-75.3)	<0.001		60.6 (25.7-82.5)		<0.001	
STAT3 constitutive (no stimulus), RFU	NA	NA	NA		NA		NA	
STAT3 (IL-6), %	98.3 (95.8-99.1)	89.3 (83.4-97.3)	0.001		95.3 (74.8-97.6)		0.006	
STAT3 (IL-6), RFU	2958 (2651-3350)	1005 (851-1802)	<0.001		2049 (1555-3041)		0.004	
Neutrophils								
STAT3 constitutive (no stimulus), %	<5 %	88.1 (34.8-95.8)	<0.001		64.4 (45.2-94.2)		<0.001	
STAT3 constitutive (no stimulus), RFU	NA	NA	NA		NA		NA	
STAT3 (IL-6), %	10.3 (8.0-14.1)	91.2 (46.8-95.6)	<0.001		76.0 (54.1-89.6)		<0.001	
STAT3 (IL-6), RFU	988 (852-1424)	1294 (1098-1459)	0.038		1562 (1407-2084)		<0.001	
Lymphocytes								
STAT1 (IL-6), %	25.3 (17.7-31.8)	6.0 (4.3-10.1)	<0.001		8.6 (4.6-11.7)		<0.001	
STAT1 (IL-6), RFU	564 (485-605) ^c	518 (425-556)	0.162		485 (397-566)		0.042	
STAT3 constitutive (no stimulus), %	<5%	61.6 (49.2-77.4)	<0.001		39.5 (34.5-50.5)		<0.001	
STAT3 constitutive (no stimulus), RFU	NA	NA	NA		NA		NA	
STAT3 (IL-6), %	54.0 (46.7-63.6)	65.2 (51.6-81.3)	0.043		48.6 (41.5-57.6)		0.156	
STAT3 (IL-6), RFU	1232 (1080-1366) ^c	1058 (916-1321)	0.092		1261 (1076-1514)		0.519	

Abbreviations: IL, Interleukin; MWU, Mann-Whitney U; NA, Not assessed; RFU, Relative fluorescence unit; STAT, Signal transducer and activator of transcription.

^abetween healthy controls and sepsis, ^bbetween healthy controls and acute pancreatitis, ^cn = 26.

Data denote median (quartiles).

Table 14. ERK1/2 MAPK signaling in ex vivo stimulated monocytes, neutrophils, and lymphocytes of healthy controls and sepsis and acute pancreatitis patients.

Leukocyte population/ Signal (stimulus), proportion (%) or RFU of positively fluorescing cells	Healthy controls, n=28	Sepsis, n=14	p value ^a , MWU	Acute pancreatitis, n=18	p value ^b , MWU
Monocytes					
ERK1/2 (PMA+A ₂₃₁₈₇), %	60.6 (34.6-78.7)	62.7 (47.6-80.9)	0.762	61.9 (29.3-77.1)	0.485
ERK1/2 (PMA+A ₂₃₁₈₇), RFU	751 (703-873)	665 (592-795)	0.030	670 (615-801)	0.030
Neutrophils					
ERK1/2 (PMA+A ₂₃₁₈₇), %	23.8 (8.2-38.5)	51.8 (33.8-58.9)	0.005	12.9 (5.4-18.7)	0.113
ERK1/2 (PMA+A ₂₃₁₈₇), RFU	987 (767-1232)	1075 (833-1189)	0.927	951 (769-1207)	0.804
Lymphocytes					
ERK1/2 (PMA+A ₂₃₁₈₇), %	40.9 (12.8-74.9)	54.8 (8.7-70.6)	0.843	13.5 (5.9-33.9)	0.009
ERK1/2 (PMA+A ₂₃₁₈₇), RFU	582 (475-679) ^c	564 (455-701)	0.790	473 (390-564)	0.045

Abbreviations: ERK, Extracellular signal-regulated kinase; MAPK, Mitogen-activated protein kinase; MWU, Mann-Whitney U; PMA, Phorbol 12-myristate 13-acetate; RFU, Relative fluorescence unit.

^abetween healthy controls and sepsis

^bbetween healthy controls and acute pancreatitis

^cn = 26

Data denote median (quartiles)

Association with the severity of acute pancreatitis

Table 15 shows the signaling pathways that (i) differed significantly between healthy controls and AP patients, and (ii) associated with the severity of AP. Of these, pSTAT1+% in monocytes and lymphocytes, promoted by IL-6, correlated inversely with the severity of AP, as did pSTAT3 RFU in monocytes. On the contrary, pSTAT3 RFU of IL-6 stimulated neutrophils increased along with the severity of AP. Additionally, constitutive STAT3 phosphorylation of unstimulated neutrophils, shown as the proportion of positively fluorescing cells, correlated with disease severity. None of the NF- κ B or ERK 1/2 signaling aberrations was associated with the severity of AP (p for trend >0.085).

Discriminating patients with organ dysfunction from those without organ dysfunction

Six signaling markers, (1) pSTAT1+% and (2) pSTAT3 RFU in IL-6 stimulated monocytes, (3) pSTAT3 RFU in IL-6 stimulated neutrophils, (4) pSTAT3+% and (5) pSTAT3 RFU in unstimulated neutrophils (constitutive activation), and (6) pSTAT1+% in IL-6 stimulated lymphocytes, were identified as potential markers in discriminating OD patients (sepsis or SAP) from those without OD (mild or moderately severe AP) (Table 16). They met the following criteria: (i) a significant difference between healthy controls and sepsis or (ii) between healthy controls and AP patients, and (iii) were associated with the severity of AP (Tables 13 and 15).

Of these, only pSTAT3 RFU of IL-6 stimulated monocytes was significantly lower in OD patients than in no-OD patients and could potentially serve as a marker (Table 16). IL-8 and HGF levels were significantly higher in OD patients than in no-OD patients.

Table 15. Leukocyte signaling aberrations associated with the severity of acute pancreatitis

Leukocyte population/ Signal (Stimulus), proportion (%) or RFU of positive cells	Severity of acute pancreatitis		p value, JT
	Mild, n=9	Moderately severe, n=6	Severe, n=3
Monocytes			
STAT1 (IL-6), %	9.1 (2.7-36.1)	4.6 (4.2-6.7)	2.2 (1.2-3.8)
STAT1 (IL-6), RFU	2371 (1692-4436)	1751 (900-3489)	1244 (693-2051)
Neutrophils			
STAT3 (IL-6), RFU	1472 (865-1831)	1988 (1441-2793)	2295 (1033-2557)
STAT3 constitutive (no stimulus), %	50.7 (14.4-80.7)	94.5 (51.0-99.1)	61.5 (47.2-99.3)
Lymphocytes			
STAT1 (IL-6), %	11.3 (1.6-42.5)	6.7 (4.5-10.6)	4.2 (3.6-5.6)

Abbreviations: IL, Interleukin; JT, Jonckheere Terpstra for trend; RFU, Relative fluorescence unit; STAT, Signal transducer and activator of transcription.

Table 16. Leukocyte signaling profiles and IL-8 and HGF levels in patients without OD and patients with OD.

Leukocyte population/Cytokine: Signal (Stimulus), proportion (%) or RFU of positive cells	No persistent OD, <i>n</i> = 15 ^a	Persistent OD, <i>n</i> = 17 ^b	<i>p</i> value, MWU
Monocytes			
STAT1 (IL-6), %	5.9 (4.4-15.1)	4.7 (3.1-6.4)	0.097
STAT3 (IL-6), RFU	2055 (1692-3456)	1065 (850-1848)	<0.001
Neutrophils			
STAT3 constitutive (no stimulus), %	67.3 (39.9-93.9)	85.5 (35.8-95.9)	0.551
STAT3 constitutive (no stimulus), RFU	1496 (1363-1634)	1296 (995-1600)	0.132
STAT3 (IL-6), RFU	1524 (1441-1893)	1314 (1086-1555)	0.058
Lymphocytes			
STAT1 (IL-6), %	10.3 (5.5-12.8)	5.6 (3.9-9.1)	0.069
Cytokines			
IL-8 (pg/mL) ^c	47.0 (15.5-114.9)	171.7 (81.0-825.6)	0.002
HGF (ng/mL) ^d	0.98 (0.71-1.74)	5.32 (2.4-23.9)	<0.001

Abbreviations: HGF, Hepatocyte growth factor; IL, Interleukin; MWU, Mann-Whitney U; OD, Organ dysfunction

^a9 with mild AP and 6 with moderately severe AP, ^b3 with severe AP and 14 with sepsis.

^cMedian (quartiles) of 24 healthy controls 9.5 pg/mL (4.0-23.5) pg/mL; *n* = 14 for no OD and *n* = 16 for OD.

^dMedian (quartiles) of 24 healthy controls 0.18 ng/mL (0.01-1.5 ng/mL); *n* = 14 for no OD and *n* = 16 for OD.

Data denote median (quartiles).

3.4 DISCUSSION

3.4.1 PREDICTING SEVERE ACUTE PANCREATITIS (I-III)

Cytokines (I, II)

Cytokines are important inflammatory mediators that play an essential part in the development of the systemic manifestations of AP, and they are detected in the circulation early after the onset of AP (Norman 1998, Mentula et al. 2005). Therefore, their ability to predict the course of AP has been of great interest since the early 1990s. Despite extensive studies on cytokines, a clinically useful marker to predict SAP remains obscure in terms of reliability, utility, and cost. Challenges in human studies include the variation between the onset of symptoms and hospital admission together with the rapid kinetics of cytokines. Indeed, the production of cytokines peaks 36-48 hours after symptom onset and then usually rapidly declines (Norman 1998). An exception may be the production of IL-8, which has been shown to continue for days or even weeks (Remick 2005). There is also a substantial heterogeneity in cytokine response between individuals (Wurfel et al. 2005). All of the above can markedly affect the cytokine levels of a single patient and make it difficult to determine reliable cut-off values to predict SAP. Combining the markers may reduce these effects, but the time-consuming analytical methods with high costs have usually restricted the number of studied cytokines to only a few at a time.

To the best of our knowledge, Study I comprises the largest number of cytokines ($n=48$) evaluated together in a group of AP patients, which was possible due to the Multiplex detection technique. This is a technique that enables multiplex screening of dozens of cytokines in small-volume samples rapidly and automatically. Here, we found that only IL-6 and HGF predicted SAP in all AP patients. This result is in accordance with previous studies since both IL-6 (Aoun et al. 2009) and HGF (Ueda et al. 1996, Espinosa et al. 2011, Sporek et al. 2013) have been demonstrated to be predictors of SAP. However, our main focus was to identify markers in the subgroup of patients without OD on admission since including also those having OD on admission most probably enhances the performance of the markers. In the current study, we found that IL-8, HGF, and G-CSF predicted SAP in AP patients without OD on admission. Of the clinical markers or symptoms, only calcium predicted SAP. Along with HGF, both IL-8 (Aoun et al. 2009) and G-CSF (Müller et al. 2000) have been shown to be predictors of SAP. However, to the best of our knowledge, this is the first study evaluating cytokines as predictors of SAP where AP patients without OD on admission were analyzed separately.

The results of Study I concerning IL-8 and HGF were further confirmed in Study II with cytokine-specific ELISA. Although in Study I the combined

logistic regression model of IL-8 and HGF provided better sensitivity than either of the markers alone, the result was not duplicated in Study II. This may be due to the significant correlation between the cytokines.

Another approach in the present investigations was to analyze the patients with OD on admission and to determine whether any of the cytokines could identify the patients with transient OD from those with persistent OD already in the early phase of AP. This approach was chosen because early OD is a rather common finding on admission to hospital, but in many patients it will resolve within the first 48 hours after admission (Buter et al. 2002, Mofidi et al. 2006). In Study II, IL-8 was shown to predict persistent OD, i.e. SAP. In Study I, the levels of HGF were higher among patients with persistent OD than in patients with transient OD, but the difference was not significant after the p value was corrected using the Bonferroni method. Moreover, these findings may not be relevant in terms of clinical practice since a patient with OD needs optimal care and monitoring preferably in the ICU, and it is not known whether the resolution of OD is due to early supportive care or the natural course of AP.

The results of Studies I and II together show IL-8 and HGF to be predictors of SAP in AP patients without OD on admission. In patients with OD on admission, IL-8 may identify those with SAP, i.e. distinguish patients with persistent OD from those with transient OD.

Nucleosomes (III)

One interesting group of potential early markers is the circulating DAMPs that are released during cell injury and promote inflammation. In Study III, we chose to evaluate the predictive value of circulating chromatin in the form of nucleosomes using a commercial ELISA assay. Although cell-free DNA has been shown to predict SAP in human AP (Kocsis et al. 2009), and elevated nucleosome levels have been found in experimental AP (Kang et al. 2014b) and in patients with sepsis (Zeerleder et al. 2003), previously, circulating nucleosome levels have not been systematically investigated in AP patients.

In Study III, we showed that nucleosome levels are associated with the severity of AP and predict SAP among all patients, and, more importantly, among those without OD on admission. The mechanisms that account for the enhanced levels of circulating nucleosomes are manifold, and their origin in AP, or in other diseases, is not yet clear, but possible sources include damaged acinar cells (Kang et al. 2014b), other injured organs, and dying neutrophils through NETosis (Marsman et al. 2016).

3.4.2 LEUKOCYTE SIGNALING IN SEPSIS AND ACUTE PANCREATITIS (IV)

Despite different triggering mechanisms, SAP and sepsis share, at least in part, common immunopathogenetic mechanisms (Wilson et al. 1998, Dib et al. 2003, Koussoulas et al. 2006), and recent papers connect the definitions of

both SAP (Banks et al. 2013) and sepsis (Singer et al. 2016) closely to the presence of (persistent) OD instead of the old and inaccurate SIRS criterion (Churpek et al. 2015). Still, AP guidelines (Working Group IAP/APA Acute Pancreatitis Guidelines 2013) suggest that the AP patients with SIRS on admission should be treated as if they have SAP. Clearly, there is an urgent need for more reliable predictive markers of SAP on admission to hospital.

The activation of intracellular signaling in blood leukocytes precedes generation of soluble mediators, such as cytokines. Therefore, it represents a very early stage of systemic inflammatory response, and the aberrations in leukocyte signaling could potentially reveal early novel markers to predict SAP or sepsis, i.e. persistent OD. The previous studies by our group (Oiva et al. 2010a, 2010b, 2013) showed multiple aberrations in signaling pathways of blood leukocytes in a cohort of SAP patients complicated by OD.

In the current study, we showed that in sepsis the aberrations in the NF- κ B, STAT1, STAT3, and ERK1/2 MAPK pathways largely resemble those discovered earlier in SAP by Oiva et al. (2010a, 2010b, 2013). The findings were similar despite the different time-points in blood sampling. While in the studies by Oiva et al. (2010a, 2010b, 2013) the sampling occurred usually only after the first hospital week, i.e. in the late phase of AP, in the current study the samples were taken within 48 hours of hospital admission. Secondly, we analyzed in a group of AP patients whether the aberrations correlated with the severity of AP, and revealed STAT1 and STAT3 as potential new predictive markers of SAP. Thirdly, we analyzed in the combined group of AP and sepsis patients whether any of the markers would distinguish patients with persistent OD from those without persistent OD and found that only IL-6-induced STAT3 phosphorylation levels in monocytes were significantly lower in the persistent OD group. The OD groups were also distinguished by IL-8 and HGF levels.

Depressed activity of the NF- κ B pathway was found in ex vivo stimulated monocytes and lymphocytes of sepsis patients relative to healthy controls, as seen in SAP with OD (Oiva et al. 2010a, 2010b). Previous studies using electrophoretic mobility shift assay of blood mononuclear cells support these findings, showing activation of NF- κ B both in sepsis (Böhrer et al. 1997, Arnalich et al. 2000, Abraham 2005) and in AP (Satoh et al. 2003, O'Reilly et al. 2006, Rakonczay et al. 2008). Although Satoh et al. (2003) showed also that NF- κ B activity was reduced in the peripheral blood mononuclear cells of SAP patients when exposed to LPS, patients but not in mild AP patients, our findings did not correlate with the severity of AP. Together, these results suggest that although NF- κ B pathway is a master regulator of inflammation and its reduced activity is a sensitive marker of systemic inflammation, it may not serve as a useful predictive marker of SAP.

Previously, IL-6 induced phosphorylation of STAT1 has been demonstrated to be reduced in activated mouse T cells (Teague et al. 2000, Van De Wiele et al. 2004). This is in accordance with the current study showing that STAT1 activity was reduced in IL-6 stimulated monocytes and lymphocytes of sepsis

patients relative to the cells of healthy controls, a finding also reported in the SAP patients of Oiva et al. (2010a, 2010b). Since STAT1 is associated with the development of Th1-mediated tissue injury, our finding may reflect a shift from Th1 to Th2-type response, which has been established to occur in both sepsis (Ferguson et al. 1999, Li et al. 2015) and AP (Ueda et al. 2002, Pietruczuk et al. 2006). In the present study, the IL-6 induced phosphorylation level of STAT1 in monocytes and the proportion of STAT1 positive lymphocytes correlated inversely with the severity of AP. Therefore, STAT1 may provide a useful predictive marker of SAP in patients with AP.

In the present study, IL-6 induced STAT3 phosphorylation was reduced in sepsis patients' monocytes, but enhanced in their neutrophils relative to healthy controls. Previously, Oiva et al. (2010a and 2013) reported similar findings in SAP. Both reduced STAT3 activity in monocytes and enhanced STAT3 activity in neutrophils correlated with the severity of AP. The opposite findings between monocytes and neutrophils may reflect more prominent anti-inflammatory or immunosuppressive mechanisms in monocytes, while the enhanced STAT3 phosphorylation in neutrophils can be explained by STAT3 activation-related priming, which has previously been described in sepsis patients (Tamassia et al. 2008).

In addition, the current study showed that STAT3 pathway was constitutively activated in sepsis patients' monocytes, neutrophils, and lymphocytes before adding a stimulus. In the studies by Oiva et al., STAT3 pathway was constantly activated in lymphocytes (2010b) and in some patients' neutrophils (2013), but not in monocytes (2010a). The different finding regarding monocytes may indicate that constitutive activation of STAT3 pathway is an early event in monocytes and it disappears as the disease progresses. In the current study, STAT3 pathway was constitutively activated also in AP patients' monocytes, neutrophils and lymphocytes, but the finding associated with the severity of AP only in neutrophils. Previously, constitutive activity of STAT3 pathway has been shown to occur in sepsis (Tamassia et al. 2008), other inflammatory conditions (Kuuliala et al. 2015), and malignant cells (Aggarwal et al. 2009). The cause may involve increased circulating levels of STAT3-activating cytokines such as IL-6 (Kuuliala et al. 2015). In contrast, in normal cells the activity of a signaling pathway is a tightly controlled mechanism with several feedback systems and interactions between different pathways, and the pathway is usually activated only for a short period of time (Aaronson and Horvath 2002, Scott et al. 2002).

Phosphorylation of ERK1/2 MAPK was reduced in PMA+A23187 stimulated monocytes in sepsis, a finding also reported by Oiva et al. (2010a) in SAP. In the present study, we did not detect an association with the severity of AP.

Taken together, we found in the current study that the signaling aberrations in the NF- κ B, STAT1, STAT3, and ERK1/2 MAPK pathways in patients with sepsis largely resemble those discovered previously in patients with SAP (Oiva et al. 2010a, 2010b, 2013). A novel finding was that the

aberrations in STAT1 and STAT3 pathways associate with the severity of AP and those in STAT3 pathway with the presence of OD. It is an intriguing question whether STAT1 and STAT3 could serve as early markers for predicting the development of OD, but this possibility requires further studies.

3.5 STRENGTHS AND LIMITATIONS OF THE STUDY

Studies I-III

The approach in Studies I-III is a novel and clinically meaningful; identifying the predictive markers of SAP in patients without OD on admission reveals the true performance of the markers in everyday hospital life. Previously, to the best of our knowledge, a similar approach has been used only in the study of Maksimow et al. (2014) showing soluble CD73 activity to predict SAP. In addition, applying various statistical methods and presenting ROC curve, AUC, sensitivity, specificity, positive and negative likelihood ratio, and diagnostic odds ratio with respective 95% confidence intervals show the predictive capacity of the markers from several angles and may be considered a methodological strength. Furthermore, choosing cut-off values with high specificity ($\geq 90\%$) is more representative of the everyday hospital life, with a constant battle for limited ICU resources, than the traditional method utilizing conventional cut-offs that maximize the sum of sensitivity and specificity.

Study limitations include at least the following: First, the number of SAP patients, especially those without OD on admission, was limited, resulting in a wide range of 95% confidence intervals and excluding any firm conclusions being drawn from the subgroup data. Secondly, the cut-off values were obtained from the same population in which their predictive value was analyzed. This post hoc analysis is known to exaggerate the results (Sternby et al. 2016); a more reliable analysis of the markers would necessitate the use of a validation cohort. Although the predictive performances of IL-8 and HGF revealed in Study I were re-evaluated in Study II using an independent cohort of AP patients, the cut-off values cannot be compared due differences in analytical methods. A third limitation is the relatively long storage time of the samples, especially in Studies I and III, before cytokine and nucleosome measurements, which may markedly affect their reported levels. Most cytokines are stable for up to 2 years of storage, but after 4 years several cytokines are degraded (de Jager et al. 2009). Long-term stability investigations of nucleosomes have revealed a 7% decrease per year in serum levels of nucleosomes during sample storage at -70°C (Holdenrieder et al. 2010). This limitation was taken into account in Study III, where we analyzed whether the storage time correlated with the nucleosome level in mild, moderately severe, or severe AP, and found no correlation.

Study IV

When interpreting the results of Study IV, their preliminary nature must be taken into account. The number of patients is very limited, consisting of only a few AP patients in different severity categories. Therefore, we could not perform a subgroup analysis among patients without OD on admission, and obviously, the associations between the altered signaling pathways and the severity of AP must be investigated in a larger group of AP patients. Additionally, the study was not planned to address questions about the ultimate pathophysiological impacts of the aberrant signaling pathways, but rather to identify possible predictive markers. Overall, leukocyte signaling constitutes a complex network of signaling pathways that work in close collaboration with each other, and the ultimate impacts of a signaling pathway via altered gene transcription are numerous. As an example, the impacts of STAT3 pathway are numerous since it activates over 3000 genes in T cells (Durant et al. 2010).

The leukocyte signaling profile determinations also have limitations. Firstly, we did not monitor cell viability. However, it is known that cell damage may increase non-specific binding of antibodies to the cell membrane (Bohn 1976), and susceptibility to membrane injury and cell viability may differ between blood leukocytes of AP or sepsis patients and healthy controls (Giamarollos-Bourboulis et al. 2006). Furthermore, cell viability and different modes of cell death, including apoptosis and necroptosis, may be associated with the severity of AP and sepsis; systematic studies are needed to explore this possibility.

3.6 FUTURE ASPECTS

Predictive biomarkers

Predicting SAP reliably on admission to hospital is an enormous challenge. One of the hurdles is the rapid evolution of OD. The incidence of OD rapidly increases by the second and third day after onset of symptoms, and about half of the patients have OD on admission to hospital or develop it during the first 24 hours after admission (Johnson et al. 2001, Mentula et al. 2003). However, the other half of SAP patients does not have OD on admission, thus constituting a clinical challenge for correct severity prediction. At the moment, the economic burden of mild AP is also substantial (Peery et al. 2015) since all AP patients need preferably at least a few days' follow-up in the hospital to determine whether AP will resolve or become complicated. Therefore, a predictive marker with reasonable accuracy is also needed for mild AP (Sternby et al. 2017).

The rapid evolution of OD encourages us to identify very early markers of systemic inflammation that would predict SAP reliably already within the first 24 hours after onset of symptoms. Besides being future potential targets for immune therapy, from a clinical perspective we should be able to identify early

the SAP patients without OD on admission to triage them accordingly. Potential novel early markers of systemic inflammation include aberrations in intracellular leukocyte signaling pathways since the activity of a signaling pathway precedes e.g. cytokine production. Also DAMPs, as they are released from damaged cells at the onset of AP and promote inflammation, have potential to serve as very early predictors. Indeed, a component of nucleosomes, namely circulating free histone levels, has been shown to rise within 2 hours after the onset of experimental AP (Ou et al. 2015), and was just recently shown to predict persistent OD with a somewhat better accuracy than IL-6 or IL-8 within 48 hours of symptom onset (Liu et al. 2017). Additionally, novel markers of systemic inflammation arising from sepsis studies, such as pentraxin 3 and soluble urokinase-type plasminogen activator receptor, have recently shown promising results as early predictors of SAP (Deng et al. 2017, Lipinski et al. 2017). Future studies re-evaluating the novel promising predictive markers of SAP should, first of all, contain large consecutive series of AP patients including a reasonable proportion of SAP patients (preferably those without OD on admission) presenting early after onset of symptoms (preferably within 24 hours).

Although we are still searching for an optimal predictive marker of SAP, it is more than likely that especially the individual differences in immune response make it virtually impossible to identify a marker that would possess clinically relevant predictive ability on its own. As improved analytical methods, such as multicytokine arrays, enable multiple detection of a large number of biomarkers simultaneously, combining them may enhance their accuracy as predictors of SAP. However, due to the significant correlations between many cytokine levels, more accurate results may be obtained by combining markers reflecting different phenomena in the pathogenesis of AP. In the future, along with constant advancements in technology and accumulating knowledge of systems biology in the fields of genomics, epigenetics, transcriptomics, proteomics, and metabolomics (as reviewed by Skibsted et al. 2013), it may well be that we will have a combination of thousands of molecular signals to help in accurate risk stratifying.

Additionally, with the increasing amount of analytical data, we will need more intelligent methods to analyze it. Besides the traditional methods, such as logistic regression models, the possibilities of a commercially available artificial neural network have already been tested in AP studies (Halonen et al. 2003, Andersson et al. 2011). Using the artificial neural network, it is possible to select from a large number of potential variables the inputs needed to create the most accurate model. Thus far, the AP studies have focused on currently available clinical and laboratory markers and the number of evaluated variables has been limited to a maximum of 23 (Andersson et al. 2011). However, the artificial neural network, as a step towards artificial intelligence, has performed better than the conventional logistic regression models.

Treatment

Currently, all AP patients are treated identically, and the interventions are symptom-related since there is no specific therapy for AP. Along with the improved understanding of the immune response during AP and sepsis, great hope has been placed on the immunomodulatory treatment as a future therapy to improve the outcome of such patients either by attenuating the early proinflammatory response or by abolishing the subsequent immunosuppression. However, the results of many clinical studies have been disappointing (Johnson et al. 2001, Kyh  l   et al. 2012), and a recent Cochrane review of pharmacological therapy in AP showed no benefits regarding mortality, nor did it find consistent clinical benefits with any intervention (Moggia et al. 2017). As already mentioned above, one of the main treatment challenges is the narrow time frame between symptom onset, hospital admission, and evolution of OD. Therefore, SAP patients without OD on admission could serve as a potential patient group for applying immune therapy. Another challenge while introducing immune therapy is that pro- and anti-inflammatory processes can occur simultaneously early in the course of disease, and not only in different compartments of the body, but also in various leukocyte subsets (Cavaillon and Annane 2006, Shi et al. 2006, Kasten et al. 2010). Additionally, we do not yet know which alterations in immune response are crucial for the outcome of disease (Venet et al. 2013). Future studies should also increase our knowledge regarding the timeframe during which the unresponsive cells will spontaneously recover their functions.

Monitoring a patient's current immune status could provide crucial information while planning the optimal treatment. Studying the phosphorylation changes in the signaling pathways of circulating leukocytes using whole-blood phosphospecific flow cytometry may bring us one step closer to this goal. Theoretically, this method could also be utilized to test the effects of a potential therapeutic agent in a whole blood sample of a patient, although it must be remembered that circulating leukocytes present only one element of the immune response, and it is possible that tissue leukocytes will respond differently to ex vivo stimulus (Caldwell and Hotchkiss 2011). In the end, it seems that applying an effective and safe immune therapy in the future invariably requires the careful immunological characterization of a single patient.

3.7 CONCLUSIONS

- I) Of the 48 evaluated cytokines, IL-8, HGF, and G-CSF serve as predictive markers of SAP in AP patients without OD on admission.
- II) The value of IL-8 and HGF as predictors of SAP was confirmed in an independent cohort of AP patients without OD on admission. IL-8 may also predict persistent OD in AP patients with OD on admission.
- II) Circulating nucleosomes predict SAP in AP patients without OD on admission.
- III) Signaling aberrations of blood leukocytes in sepsis resemble those discovered earlier in SAP. Aberrations in STAT1 and STAT3 pathways, but not in NF- κ B, may be associated with the severity of AP. Aberrations in STAT3 pathway may also be associated with OD.

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